DOCTOR OF PHILOSOPHY

Characterisation of the sulfotransferases catalysing the sulfation of xenobiotics and steroids in bovine liver

Choughule, Kanika

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Kanika Choughule

Thesis submitted for the degree of
Doctor of Philosophy at the
University of Dundee

October 2011

Declaration

I declare that this thesis is based on results gained from investigations that I have personally conducted and that this thesis is of my own composition. Any work other than my own is clearly indicated in the text with reference to any publications from relevant researchers. This thesis has not been previously presented, in whole or in part for a higher degree.

Kanika Choughule

I confirm that Kanika Choughule has spent the equivalent of at least 9 terms in research work in the Division of Medical Sciences, Ninewells Hospital and medical School, University of Dundee. She has fulfilled the conditions of the University of Dundee thereby qualifying her to submit this thesis in application for the degree of Doctor of Philosophy.

Professor Michael W.H. Coughtrie

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Abbreviations

ADP	Adenosine di phosphate
APS	Ammonium persulfate
ATP	Adenosine tri phosphate
BSA	Bovine Serum Albumin
bSULT	Bovine sulfotransferase
cDNA	Complementary DNA
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
dH_2O	Distilled water
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
GI	Gastrointestinal
GST	Glutathione S-transferase
HEPES	4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMM	Hepatocyte maintenance media
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Broth
MAP	Multiple antigenic peptide
MBP	Maltose binding protein
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
N-OH-pHIP	2- (Hydroxyamino)-1-methyl-6-phenylimidazo[4,5-b]pyridine
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PSB	Phosphosulfate binding loop
SULT	Sulfotransferase
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
UGT	Uridine 5'-diphospho-glucuronosyltransferase
UPLC	Ultra Performance Liquid Chromatography

Abstract

Cattle are a very important part of the human food chain. Administration of veterinary drugs and other xenobiotic compounds to cattle can often result in the accumulation of metabolic residues in edible tissues that can potentially affect humans through the food chain. An understanding of drug metabolism in this species is vital to ensure safe use of drugs in cattle and eventually the provision of safer animal derived food products to man. Sulfation catalysed by sulfotransferases (SULTs) is an important phase 2 drug metabolising reaction. It is not only involved in the detoxification of drugs and xenobiotics but also in the bioactivation of procarcinogens. It is also important in the metabolism of several drugs used routinely in cattle. However, very little work has been carried out on SULTs in cattle. A variety of *in vitro* tools are available to study drug metabolising enzymes (DMEs) like SULTs. These include tissue microsomes, cytosol, recombinant enzymes and isolated cells such as the hepatocytes. Recombinant SULTs are important tools that can be used for the study of isoform specific drug biotransformation, drug-drug interactions and the effect of genetic polymorphisms on the activity of specific isoforms. As the liver is the major drug metabolising organ in the body, it is essential to study expression and activity of cytosolic liver sulfotransferases. Hepatocytes contain DMEs and drug transporters along with all the necessary cofactors that represent *in vivo* conditions. This makes hepatocytes a better representative of *in vivo* conditions as compared to microsomes, cytosol or recombinant enzymes. In this study we have characterised sulfotransferases in cytosol, recombinant enzymes and hepatocytes.

Antibodies previously raised against human sulfotransferase isoforms were used in the detection of cytosolic bovine sulfotransferases. Probe substrates established for activity with human SULTs were used for assessing the activity of recombinant and cytosolic bovine sulfotransferases. Cytosol was prepared from 8 male livers and 12 female livers (8 untreated and 4 treated with an exogenous progestin). SULT1B1, SULT1E1 and SULT2A1 were detected in bovine liver cytosol. Expression of SULT2A1 in the bovine liver was sex specific with males expressing almost twice as much SULT2A1 compared to the females. However, no activity was detected with dehydroepiandrosterone (DHEA) which is used as a probe substrate for SULT2A1 in humans. Pregnenolone is metabolised by SULT2A1 and SULT2B1 in humans. Activity towards this substrate was detected in the bovine liver, however no sex related differences in activity were observed. 4-nitrophenol is metabolised by several members of the SULT1 family in humans such as SULT1A1, SULT1B1 and SULT1C. 17β-estradiol is a probe substrate for human SULT1E1. Activity was detected with 4-nitrophenol in male and female bovine livers. Male liver cytosol followed Michaelis-Menten kinetics whereas the female liver cytosol displayed partial substrate inhibition. This suggests that different enzymes have been involved in the biotransformation of 4-nitrophenol in the male and female liver. Activity towards 17β-estradiol in the female liver was almost 4 times higher than in the male liver.

Recombinant bovine sulfotransferases (SULT1A1, SULT1B1, SULT1E1 and SULT2A1) were expressed in *E. coli.* All bovine SULTs except SULT2A1 were expressed in the soluble fraction. Like human SULT1A1, bovine SULT1A1 also displayed partial substrate inhibition, however the extent of inhibition (as seen with the K_i values) was lower compared to human SULT1A1. Bovine SULT1B1

followed Michaelis-Menten kinetics with 4-nitrophenol. Substrate specificity profiling carried out with equal amounts of bovine SULT1A1 and SULT1B1 revealed that SULT1B1 was better at sulfating phenolic compounds as compared to bovine SULT1A1. SULT1A1 is highly expressed in the human liver and is the major enzyme involved in drug metabolism in the human liver. This might not be the case in cattle given that SULT1B1 was found to be better at sulfation than SULT1A1 and expression of SULT1A1 was not detected in the bovine liver using antibodies. Human SULT1E1 is known to metabolise 17β-estradiol with a very high affinity and with a K_m in the low nanomolar range. Comparatively, bovine SULT1E1 metabolised 17β-estradiol with a lower affinity, in the micromolar range.

Expression and activity of bovine sulfotransferases differed from human sulfotransferases and some of the differences could be attributed to key amino acid residue substitutions in the active site of the bovine SULTs. For example, substitution of Phe141 in human SULT1E1 to Leu141 in bovine SULT1E1 restricts the ability of bovine SULT1E1 to form strong van der Waals interactions with the substrate due to loss of an aromatic hydrocarbon ring. This could explain the reduced affinity of bovine SULT1E1 for 17β-estradiol. Substitutions of small uncharged residues with large charged ones in the active site of bovine SULT2A1 could have unforeseeable effects that could result in the formation of an insoluble protein. Substitutions in the active site of bovine SULT1A1 that bind the second molecule of 4-nitrophenol could be responsible for the reduced partial substrate inhibition effects observed in comparison to human SULT1A1. In order to further validate some of these findings it would be necessary to perform additional experiments that involve mutating the substituted residue to the original one as

found in the human/mouse counterpart and looking for restoration of original properties.

The work was extended to investigate conjugative metabolism of the steroid hormone 17β-estradiol and its stereoisomer 17α-estradiol in microsomes, cytosol and cryopreserved hepatocytes all prepared from bovine liver. It was found that glucuronidation was the main route for estradiol metabolism in cattle since large amount of glucuronide metabolites were detected in microsomes and cryopreserved hepatocytes. In comparison no sulfate metabolites were detected in cytosol and hepatocytes.

We now have a better understanding of some of the important phase 2 drug metabolism pathways in cattle.

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1 Introduction

1.1 Animal pharmaceutical market and regulation

The worldwide animal health market was estimated at $11 billion in sales in 2001 with Northern America and Western Europe accounting for 60% of the market (Evans and Chapple, 2002). Livestock products dominated the market as they accounted for 70 % of the sales with cattle health products accounting for 31.1% followed by companion animals at 29.3%, pigs (19%), poultry (14%) and sheep (7%) (See figure 1). Since 1991, the companion animal market has grown at 6.6% per annum. This matches the growth seen for the human healthcare market (Ahmed and Kasraian, 2002). The prime reasons for this are the economic growth in the developed world in the 1990s and pet owners' willingness to invest in the health of their animals. This coupled to lower costs, risk and improved time to market makes it more profitable compared to food producing animal products. However, the companion animal industry as opposed to the livestock industry is more vulnerable to changes in the economic situation and hence the pharmaceutical industry still continues to invest in food producing animals (Ahmed and Kasraian, 2002). Since 1991, the animal health industry has grown by 1.9% per annum as opposed to the human health industry which is touching a double digit growth. In contrast to the 'blockbuster drug' oriented human healthcare industry, the animal health industry consists of a large number of drugs with small revenues. Indeed only 17 animal health products have sales that exceed $100million in the US and only 6 products in the UK have annual sales exceeding £5million. Infact, 93% of all licensed animal healthcare products in the UK have annual sales of less than £1million (Evans and Chapple, 2002). Developing human health care products is a longer and a more stringent process as compared to the development of animal healthcare products. The former requires preclinical

testing in animal models followed by phase 1 trials to evaluate safety and phase 2 and 3 to confirm safety and efficacy. In contrast, animal healthcare products require establishment of safety and efficacy in the target species eliminating the need to carry out preclinical and phase 1 study. The time to reach the market is 2-3 years shorter compared to human healthcare products. In addition to this there is little or no post-marketing surveillance (phase IV) for animal healthcare products (Ahmed and Kasraian, 2002).

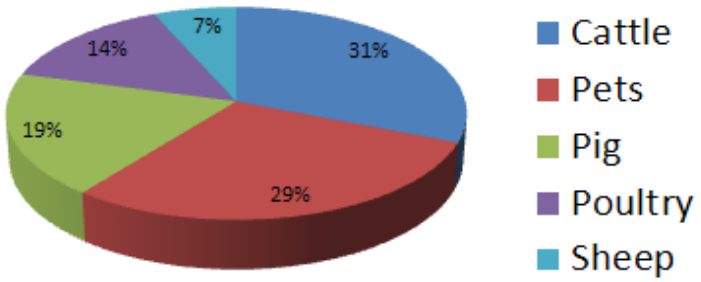

Figure 1: The animal pharmaceutical market

Market shares of cattle, pets, pigs, poultry and sheep in the animal pharmaceutical market. (Evans and Chapple, 2002)

1.2 Concern over drug residues in animal derived food products

Contamination of animal derived food products is a major cause of concern to the animal health industry. Chemical safety of animal derived food products is required to ensure consumer health and to maintain consumer confidence and satisfaction. Contamination of animal food products can occur by the presence of inorganic residues such as mercury, arsenic, lead and cadmium or organic

residues such as dioxins (Andree et al., 2010). However, exceedingly relevant and of great concern to the animal pharmaceutical industry and regulatory bodies is the presence of metabolic residues of veterinary drugs and growth promoters in animal derived food products (O'Keeffe, 1998; Reig, 2008; Stolker, 2007).

Clenbuterol is a β2-adrenergic agonist that has been traditionally used as a bronchodilator in people with breathing disorders. It can also cause an increase in aerobic capacity, central nervous system stimulation and increase in blood pressure and oxygen transportation. It is also used illegally as a performance enhancing drug by athletes. In animals, it was used as a treatment for respiratory disorders. It was also used as a muscle relaxant in cattle. Its ability to increase muscle-to-fat ratio makes it a popular option to use to obtain leaner meats illegally in cattle. In addition to this it was also used a non-steroidal anabolic growth promoter. Recent incidences of clenbuterol poisoning in Portugal, and China have been reported by consumption of lamb and bovine meat containing metabolic residues of clenbuterol. Intoxicated people developed symptoms such as nausea, headache, tachycardia and myocardial infarctions (Barbosa, 2005). Use of clenbuterol is now banned in the US and Europe (Mitchell and Dunnavan, 1998). The presence of residual antibiotics in edible tissues can cause allergies in humans or the transfer of resistant bacteria through the food chain (Butaye et al., 2001). Ingestion of trace levels of antimicrobials can disturb the composition of human intestinal gut flora which acts as a barrier to colonization from pathogenic bacteria and hence aids digestion (Cerniglia and Kotarski, 1999). Furazolidone, nitrofurazone and nitrofurantoin are nitrofuran antibacterial agents which have been used widely as feed additives for the treatment of gastrointestinal infections in cattle, pigs and poultry. Use of nitrofurans in the EU was prohibited in 1997

after research showed that furazolidone was a mutagenic and genotoxic drug. In addition to this, natural hormones such as 17β-estradiol, testosterone and synthetic steroids such as estradiol benzoate, trenbolone acetate that are administered to improve growth rate in animals are also known to produce metabolites of a carcinogenic potential. Their usage in food producing animals is strictly regulated (Andree et al., 2010). Synthetic derivatives of natural steroid hormones such as melengestrol acetate (progesterone) are used for oestrus synchronisation in cattle.

In the United Kingdom alone, adverse drug reactions (ADRs) in humans account for 7% of hospital admissions of which approximately 0.15% result in death. This costs the national health services (NHS) in excess of £400 million per year (Pirmohamed et al., 2004). In order to minimize ADRs in humans as a result of consuming contaminated animal food products, it is important to understand the process of drug and xenobiotic compound metabolism in food producing animals.

In the past, research has mainly focussed on understanding drug metabolism in laboratory animals such as rat, mouse and guinea pig and extrapolation of metabolism data from laboratory animals to livestock species (Friess, 1983; Rumsey, 1983; Watkins and Klaassen, 1986). Marked differences exist in the extent of metabolism and nature of metabolites formed across species (Dalvi et al., 1987; Nebbia et al., 2003; Short, 1994). Studies relating hepatic biotransformation of xenobiotics reported 100-fold to 1000-fold differences in enzyme activities towards certain substrates measured under identical conditions in laboratory animals (Gregus et al., 1983) and livestock species (Smith et al., 1984). In light of these differences observed, it is essential to have drug metabolism data on animals

of the livestock species, especially cattle which is one of the major food producing animals.

1.3 Drug Metabolism

Drug metabolism can be defined as the biochemical modification of xenobiotic compounds by a specialised group of enzyme system present in the liver, kidney, lung and the GI tract (Meyer, 1996). The endoplasmic reticulum in the liver is the major site of drug metabolism. Drug metabolism can result in either the production of toxic (activation) or nontoxic metabolites (deactivation) although majority of the final drug metabolite is water soluble detoxified product that is rapidly excreted through urine and bile (See figure 2). Drug metabolism usually proceeds through phase 1 and phase 2 reactions. Phase 1 reactions generally precede phase 2 reactions although they can also occur independently of each other. Phase 1 reactions are involved in the functionalization of drugs and xenobiotic compounds which often increases their biological activity. Phase I reactions may occur by oxidation, reduction, hydrolysis, cyclization or decyclization reactions (Meyer, 1996). The majority of the phase 1 reactions are carried out by a group of enzymes called the cytochrome P450s (CYPs). Oxidation reactions brought about by the CYP enzyme family discovered in the 1950s are the most extensively studied Phase I reactions. CYPs are membrane bound enzymes found in the endoplasmic reticulum or the surface of mitochondria. CYPs are a large family of phase 1 drug metabolising enzymes with 18 families and 48 subfamilies. If the drug molecule is sufficiently polar past the phase 1 stage it can be excreted, however this is generally not the case. In phase 2, polar functional groups of phase 1 metabolites are conjugated with endogenous substrates to form highly polar conjugates which are readily excreted. Metabolites of phase 2

reactions are usually too hydrophilic to diffuse out of the cell on their own and need to be actively pumped out. This job is done by some members of the ATP-binding cassette (ABC) transporter family such as multi drug resistant associated proteins (MRP2), breast cancer resistant protein (BCRP) and P-glycoprotein (pgp). These transporters are referred to as phase 3 metabolism.

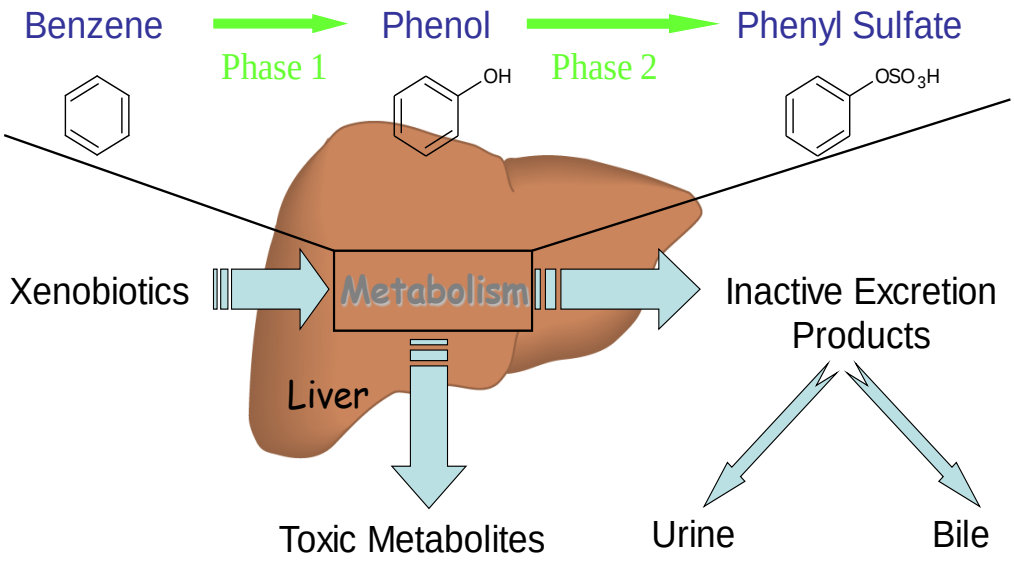

Figure 2: Schematic representation of xenobiotic/drug metabolism in liver.

Benzene is oxidised to phenol in phase 1 and subsequently conjugated to a sulfate group in phase 2. Metabolism can give rise to toxic and active as well as nontoxic and inactive metabolites although the latter is more often the case.

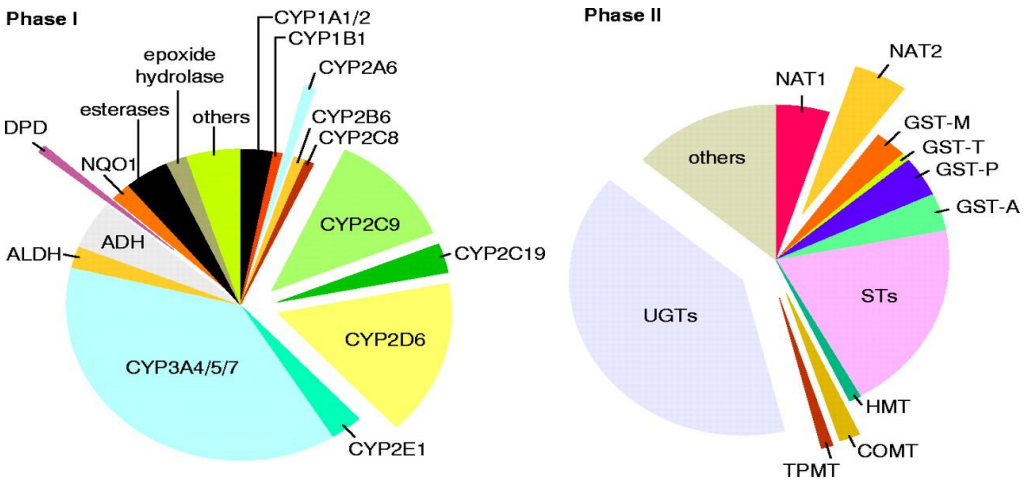

Figure 3: The percentage of phase I and phase II metabolism of drugs contributed by each enzyme in the human liver.

It is estimated by the relative size of each section of the corresponding chart. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; CYP, cytochrome P450; DPD, dihydropyrimidine dehydrogenase; NQO1, NADPH:quinone oxidoreductase or DT diaphorase; COMT, catechol O-methyltransferase; GST, glutathioneS-transferase; HMT, histamine methyltransferase; NAT,N-acetyltransferase; STs, sulfotransferases; TPMT, thiopurine methyltransferase; UGTs, uridine 5′-triphosphate glucuronosyltransferases. (Evans and Relling 1999).

1.4 Drug Metabolism in Cattle

Most of the research carried out on drug and xenobiotic metabolism in cattle has involved cytochrome P450s. Very little research has been done on phase 2 drug metabolism in cattle. In the following section I present current knowledge on drug metabolism in cattle. Cytochrome P450 (CYP) enzymes represent a superfamily of membrane bound haem containing mono-oxygenases that catalyse the incorporation of an atom of oxygen into the substrate from molecular oxygen using the co-factor NADPH. These enzymes are present in almost all living

organisms from bacteria to mammals and are involved in the activation/detoxification of xenobiotics, endogenous steroids and pharmaceutical drugs (Fink-Gremmels, 2008). They have been extensively studied in humans and laboratory species such as the rat. Relatively less research has been carried out on food producing species including cattle. Most of the studies have used rat and human as a reference species i.e. using rat/human antibodies for the detection of CYPs and rat/human probe substrates for expression and activity studies respectively in livestock animals (Darwish, 2010; Machala et al., 2003; Sivapathasundaram et al., 2001; Szotakova et al., 2004). In veterinary species, CYPs are most abundant in liver followed by the lung, kidney and intestine. Members of the CYP1A, 2B, 2C, 2D,2E, 3A and 4A have been detected in the liver of cattle (Grasso et al., 2005). CYP1A1 expression in mostly hepatic in humans and rats (Ioannides and Parke, 1990) but in the bovine it is predominantly extrahepatic and was found to have higher activity than ovine, porcine and caprine liver microsomes for ethoxyresorufin O-deethylation (EROD)(Darwish, 2010), the classic CYP1A1 probe substrate (Szotakova et al., 2004). CYP1A1 catalyses the biotransformation of a variety of environmental pollutants and veterinary drugs (Ioannides and Parke, 1990). Unlike CYP1 family, the contribution of CYP2 family members to drug metabolism is modest unless induced by appropriate compounds. For example benzphetamine and 16α-testosterone are CYP2B6 substrates in man and rat (Ioannides, 2006). In cattle, a 3 fold increase in 16β-OH testosterone production was observed in liver microsomes treated with phenobarbital (Cantiello M, 2006). CYP2B enzymes metabolise small molecule drugs such as barbiturates, benzodiazepines or pesticides such as aldrin and parathion (Nebbia et al., 2003). In the bovine, CYP2B6 is highly expressed in the lung as compared to other tissues (Darwish,

2010). However other members of the CYP2 family like CYP2C9 and CYP2E1 have a strong hepatic expression (Darwish, 2010). CYP2E1 is also induced by xenobiotics like acetone and alcohol. The CYP3 family is the most active contributor to drug metabolism and is responsible for the metabolism of a wide range of xenobiotics, steroids and medicinal drugs like chlorpheniramine, chlorpromazine, erythromycin and bromhexine. The *N*-demethylation of the antibiotic erythromycin is used as a probe for studying the activity of CYP3A4 in humans and rats (Wrighton et al., 1985). High expression of CYP3A4 was recorded in cattle liver as compared to other drug metabolising organs (Darwish, 2010). In contrast to CYP1, 2 and 3 families which are mainly involved in the metabolism of xenobiotics and pharmaceutical drugs, CYP4 family members are involved in the oxidation of fatty acids. CYP4A activity in humans and rats is measured using ω-hydroxylation of lauric acid (Tamburini et al., 1984). It was found that bovine liver microsomes poorly catalysed this substrate in comparison to rat, sheep pig and goat. The amount of immunoreactive protein detected was also lower in comparison to rats (Szotakova et al., 2004).

Glutathione S-transferase (GSTs) is a phase 2 enzyme multigene family involved in the detoxification of various exo and endogenous compounds such as plant phenols, mycotoxins and carcinogens. They are also involved in steroid metabolism and prostaglandin synthesis (Sheehan et al., 2001). GSTs catalyse the nucleophillic attack of GSH on electrophilic substrates (Gusson et al., 2006). To date 7 classes of GSTs have been identified in mammals (Frova, 2006). GSTα contributes significantly to the biotransformation of promutagens and procarcinogens. 1-chloro-2,4-dinitrobenzene (CDNB) is a substrate widely used for assessing GSTα activity (Gusson et al., 2006). Activity of cattle towards this

substrate was lower when compared to that of rats and other species such as rabbits, horses and pigs (Gusson et al., 2006; Sivapathasundaram et al., 2003). GST-CDNB activity in rat hepatocytes was found to be 6 fold higher than bovine hepatocytes and this has been inversely related to different hepatic cytotoxic potency of aflotoxin B1 from either species (Kuilman et al., 2000). Moreover cattle were also found to be supposedly deficient in other classes of GST enzymes (Gusson et al., 2006). Expression of GSTα like mRNA has been detected in liver, lung, kidney and muscle tissue of cows with the kidney having the highest expression levels of all the tissues examined (Darwish, 2010).

Several factors such as breed, sex and age affect the expression profile and activity of drug metabolising enzymes in the above animals including cattle (Giantin et al., 2008) and these factors must be taken into account when comparing drug metabolism data between animals.

1.5 Sulfation

Sulfation has been known to be involved in the modification of several compounds that carry out diverse physiological processes. These include endogenous molecules such as steroid hormones (Luu-The et al., 1996), cholesterol, low molecular weight compounds such as iodothyronies (Visser, 1994; Visser et al., 1998) and vitamin C (Baker et al., 1971) as well as large macromolecules such as proteoglycans found in connective tissue (Bernfield et al., 1999; Kolset and Salmivirta, 1999). Posttranslational modification of secreted and membrane bound proteins by sulfation has generated a lot of interest in sulfation being implicated in protein-protein interaction mediating various biological process such as leucocyte adhesion, chemokine signalling and homeostasis (Kehoe and Bertozzi, 2000). It is also involved in the biotransformation of various

endogenous and xenobiotic compounds. Some very commonly used human drugs metabolised by sulfation include paracetamol, minoxidil and salbutamol (Anderson et al., 1998; Clements et al., 1984; Zhang et al., 2007). Sulfation was first discovered by Eugen Baumann in 1876 when he isolated and characterized the sulfate conjugate of phenol from urine of a patient treated with carbolic acid as an antiseptic. However, it was not until 1953 when the mechanism of biotransformation by sulfation was elucidated. In 1953, Lipmann et al discovered 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the obligatory co-factor / sulfate donor of sulfation reactions (Robbins and Lipmann, 1957). Sulfation is defined as the transfer of sulfate radical (SO^{3-}) from a donor molecule 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to an acceptor site on the substrate (Klaassen and Boles, 1997). Given below is an illustration of the sulfation reaction. PAPS is synthesised from an inorganic sulfate and two molecules of ATP and hence is an expensive molecule for the cells to make. It is present in low concentration in the cell (<20-30μM). Sulfation is limited by the bioavailability of PAPS (Falany, 1997). The synthesis of PAPS from inorganic sulfate results from the concerted action of two enzyme systems namely, ATP sulfurylase and APS kinase. The first step in PAPS synthesis is catalysed by ATP sulfurylase and it involves the reaction of ATP and inorganic sulfate (SO_4^{2-}) to form adenosine 5'-phosphosulfate (APS) and pyrophosphate (PPi) in the presence of Mg^{2+}. In the second step, APS reacts with another molecule of ATP to form PAPS and ADP. This reaction too takes place in the presence of Mg^{2+} but is catalysed by APS kinase (Lyle et al., 1994a; Lyle et al., 1994b) (See figure 4). In most species however, these 2 enzyme systems are incorporated in a single bifunctional protein called the PAPS synthetase. The catalytic domain of APS kinase is located at the N terminal region whereas the ATP sulfurylase domain is located at the C terminal end of this bifunctional protein

(Venkatachalam et al., 1998). In humans and mice, 2 isoforms of this enzyme exist that are products of genes *PAPSS1* and *PAPSS2*. Mutations in PAPSS2 are responsible for a rare inherited connective tissue disorder in humans called spondyloepimetaphyseal dysplasia (Faiyaz ul Haque et al., 1998).

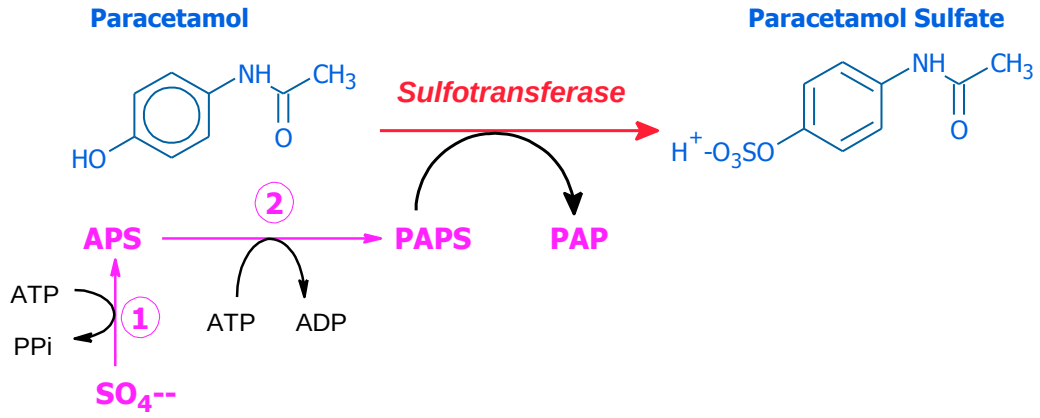

Figure 4: Sulfotransferase catalysed sulfation of the drug paracetamol.

The reaction depends on the availability of the obligate co-factor PAPS. PAPS is synthesized in a two stage reaction (1 and 2) and involves the use of an inorganic sulfate and 2 molecules of ATP (Coughtrie, 2002)

1.6 The Sulfotransferase enzyme superfamily

Sulfation is carried out by two broad classes of enzyme families termed as sulfotransferases (SULT). Membrane bound SULTs are located in the golgi apparatus of the cell and sulfate large macromolecules such as proteins, lipids and glycosaminoglycans. On the other hand, cytosolic SULTs sulfate xenobiotics and small endogenous compounds such as steroids, bile acids and neurotransmitters (Falany, 1997; Negishi et al., 2001). The focus of this research is limited to studying cytosolic sulfotransferases only. Like many other drug metabolising enzymes sulfotransferases belong to a large family of enzymes. Attendees at the third international sulfation workshop held in Drymen, UK (1996) and the fourth

international ISSX meeting in Seattle (1995) agreed that 'SULT 'should be adopted as the abbreviation for cytosolic sulfotransferases and that '*SULT* 'would be the gene symbol. Nomenclature published by Blanchard et al, puts SULTs sharing 45% sequence identity into the same family and members within the same family sharing more than 60% identity are put in the same sub family. cDNAs that encoded amino acids with more than 97% sequence identity were assigned identical isoform names. SULT families were designated by an Arabic numeral following the name (e.g. SULT1) and sub families recognized by alphabetical letters (e.g. SULT1A) (Blanchard et al., 2004). Isoforms within a subfamily were recognized by an Arabic numeral following the subfamily alphabet. (e.g. SULT1A1). It was decided that the first published sequence in a subfamily would be recognized as isoform 1 and any other isoforms discovered subsequently would be assigned Arabic numbers based on their percentage amino acid identity relative to isoform 1.

1.6.1 SULT1A family

The SULT1 family is subdivided into 5 subfamilies. Members of the SULT1A family are also known as phenol sulfotransferases due to their ability to primarily sulfate small phenolic compounds (Brix et al., 1999; Wilborn et al., 1993). However, their sulfation capacity is not limited to small phenolic compounds as they can also sulfate endogenous compounds such as 17β-estradiol, 17α-ethinylestradiol (Falany, 1997) and iodothyronines (Li et al., 2001) and carcinogens such as N-hydroxy-PhIP (Ozawa et al., 1995). SULT1A1 has been identified in a large number of species including humans (Wilborn et al., 1993), rat (Ozawa et al., 1990), mouse (Kong et al., 1993), cow (Schauss et al., 1995) and rabbit (Riley et al., 2002). In most of the species only a single SULT1A member, SULT1A1 has been identified.

In humans, 4 SULT1A genes namely *SULT1A1*, *SULT1A2*, *SULT1A3* and *SULT1A4* have been identified (Blanchard et al., 2004; Hildebrandt et al., 2004). Genes encoding these proteins are clustered around the short arm of chromosome 16 and seem to have arisen due to gene duplication or recombination events (Dooley, 1998). Human SULT1A1 was first characterized following isolation from liver cDNA library. The protein exists as a dimer in solution, is made up of 295 amino acid and has a subunit molecular mass of 32KDa (Wilborn et al., 1993). It is widely distributed throughout the body with the highest expression in liver and expression to a lesser degree in lung, brain (Richard et al., 2001), skin, breast (Falany and Falany, 1996a), intestine (Teubner et al., 2007) kidney, placenta and platelets (Gamage et al., 2006). Human SULT1A1 is also the most extensively studied isoform with respect to biotransformation of several drugs such as paracetamol, and minoxidil (Gamage et al., 2006; Meisheri et al., 1993). Studies carried out at the mRNA level show that SULT1A1 is widely expressed in human drug metabolising tissues. Protein quantification studies have shown that SULT1A1 is the most abundant enzyme present in human liver (Riches et al., 2009). Because of its broad tissue distribution, broad substrate specificity and high expression in major drug metabolising tissues such as the liver and GI tract, SULT1A1 is considered the major drug/xenobiotic metabolising SULT.

Ozawa *et al* first cloned the SULT1A2 cDNA from a human liver library. It has approximately 95% sequence identity to SULT1A1 (Ozawa et al., 1995). This isoform has only been identified in humans. It sulfates phenolic substrates such as 4-nitrophenol, 2-naphthol and minoxidil with a much lower affinity than SULT1A1 (Ozawa et al., 1995). Although SULT1A2 cDNA has been isolated from human liver (Zhu et al., 1996), to date protein expression of SULT1A2 has not been detected in

any human tissue. Due to low levels of activity and no expression relative to SULT1A1, this isoform is not considered important with respect to drug/xenobiotic metabolism.

SULT1A3 was initially isolated from a human brain cDNA library (Zhu et al., 1993). It encoded a 295 amino acid peptide that resulted in the formation of a 34kDa protein that was 93% similar to SULT1A1 (Zhu et al., 1993). Although the sequence identity of the whole gene was 60%, the coding region of SULT1A3 had > 90% sequence identity to SULT1A1 (Blanchard et al., 2004). SULT1A3 is not typically a phenol sulfotransferase like SULT1A1; instead it specifically sulfates catecholamines and neurotransmitters such as dopamine (Dajani et al., 1999b). *SULT1A3* and *SULT1A4* produce identical proteins that can be referred to as SULT1A3 or SULT1A4. It will be referred to as SULT1A3 throughout this manuscript. It is believed that *SULT1A3* and *SULT1A4* must have arisen from sequential gene duplication on chromosome 16 (Hildebrandt et al., 2004). Gene (s) encoding SULT1A3 has thus far been identified only in primates. SULT1A3 is expressed in human brain, intestine, placenta and lungs (Richard et al., 2001; Windmill et al., 1998). It is majorly expressed in the fetal liver; however no expression has been detected in the adult liver.

1.6.2 SULT1B

The first member of this family was cloned from a rat liver cDNA library and was shown to have activity towards tyrosine substrates and L-dopa (Sakakibara et al., 1995). The first human form of SULT1B1 was isolated and characterized from a liver cDNA library (Fujita et al., 1997; Wang et al., 1998). The human form of SULT1B1 was shown to be 74% identical to rat SULT1B1. The structural gene for SULT1B1 is located on 4q13.1 (Meinl and Glatt, 2001) and encodes a protein that

is 296 amino acids long and has a subunit molecular mass of approximately 35KDa. SULT1B1 was initially termed as the thyroid sulfating enzyme because of its high affinity to thyroid hormone substrates and its ability to sulfate a range of thyroid hormones (Wang et al., 1998). Indeed human SULT1B1 may be the principal hepatic iodothyronine sulfating enzyme. Expressed SULT1B1 has also been shown to conjugate phenolic substrates such as 1-naphthol, 4-isopropylcatechol and 4-nitrophenol. Overlapping substrate specificities with SULT1A1 make the identification of a probe substrate that is exclusively metabolised by SULT1B1 very difficult. Most steroids are not readily sulfated by SULT1B1 (Fujita et al., 1999). Expression of human SULT1B1 has been detected in liver, colon, small intestine and leukocytes. No expression in thyroid tissue has yet been determined. In addition to rats and humans, the SULT1B1 enzyme has been isolated and characterized from mouse, chicken (Wilson et al., 2004) and dog (Blanchard et al., 2004)

1.6.3 SULT1C

Members of the SULT1C family have been identified in a variety of species such as Sult1C1, 1C2 and 1C3 from rat, Sult1C1 from mouse and Sult1C2 from rabbit (Blanchard et al., 2004) and chicken SULT1C1 (Wilson et al., 2004). In humans, SULT1C1, 1C2 and 1C3 members of the family have been identified. Human SULT1C2 was first isolated from a fetal liver spleen library (Freimuth et al., 2000) . *SULT1C2* is located at chromosomal position 2q11.2 (Her et al., 1997) and encodes a protein that is 302 amino acids long. *SULT1C1* is also located at position 2q11.1-11.2 and encodes a 296 amino acid long protein that is 63% identical in sequence to SULT1C2 (Nagata et al., 1993). Expression of human SULT1C1 and 1C2 was detected in fetal liver and kidney (Her et al., 1997). In addition to this, 1C2 was

also found to be expressed in adult human stomach and kidney (Her et al., 1997). Human SULT1C2 has been shown to catalyse the sulfation of high concentration of 4-nitrophenol as well as metabolically activating procarcinogens such as N-hydroxy-2-acetylaminofluorene (Freimuth et al., 2000). Chicken SULT1C1 had activity towards 4-nitrophenol, 2-bromophenol, dopamine, tyrosine and apomorphine (Wilson et al., 2004).

1.6.4 SULT1E

SULT1E1 is most widely studied for its role in estrogen biotransformation. Human SULT1E1 has a very high affinity (in the nanomolar range) towards natural hormones such as 17β-estradiol and estrone. It also sulfates a variety of synthetic estrogens such as 17α-ethinylestradiol, diethylstilbesterol and 4-hydroxytamoxifen (Falany and Falany, 1997). Although other sulfotransferases such as SULT1A1 and SULT2A1 also metabolise 17β-estradiol, they do so at non-physiological conditions i.e. in the micromolar range (Falany and Falany, 1997). Bovine SULT1E1 was the first cDNA cloned as a known sulfotransferase (Nash et al., 1988). Since then members of the SULT1E subfamily have been identified in guinea pig, rat, mouse, pig and humans (Aksoy et al., 1994; Blanchard et al., 2004). Human SULT1E1 is expressed in fetal and adult liver, jejunum, lung, kidney and hormone responsive tissues such as the endometrium, breast, testis and placenta (Coughtrie, 2002). The enzyme is expressed in the endometrium and is tightly regulated during the menstrual cycle under the influence of progesterone (Buirchell and Hahnel, 1975; Falany and Falany, 1996b; Rubin et al., 1999). In addition to estrogenic compounds, SULT1E1 is also known to be a principal SULT involved in thyroxine metabolism (Kester et al., 1999a).

1.6.5 SULT2A family

Members of the SULT2 family are commonly referred to as hydroxysteroid sulfotransferases due to their ability to sulfate a wide range of steroids. The SULT2 family has been divided into 2 subfamilies termed SULT2A and SULT2B based on their amino acid sequence identities (Blanchard et al., 2004). SULT2A1 is commonly referred to as dehydroepiandrosterone (DHEA) SULT due to DHEA being its preferred substrate. However in addition to DHEA, the enzyme also metabolises other steroids such as pregnenolone, testosterone, estradiol, estrone and androsterone (Comer et al., 1993; Forbes et al., 1995). SULT2A2 was first identified in rats who are known to have 3 different isoforms of SULT2A namely SULT2A1 (Ogura et al., 1989), SULT2A2 (Chatterjee et al., 1987) and SULT2A3 (Ogura et al., 1990). SULT2A1 has also been identified in various other species such as the mouse, rabbit, humans and monkey (Blanchard et al., 2004). As opposed to the 3 SULT2A isoforms discovered in rats, only a single SULT2A1 isoform has thus far been identified in humans. In humans, SULT2A1 is primarily expressed in liver, GI tract, steroidogenic organs such as adrenal and ovary and androgen dependent tissues such as prostrate (Javitt et al., 2001). SULT2A1 is known to be an important bile acid sulfating enzyme in the human liver where it functions to protect the liver against the hepatotoxic effects of bile acids (Radominska et al., 1990). In rats it has emerged that SULT2A1 might play an important role in androgen responsiveness of the liver during development by controlling the sulfoconjugation of testosterone (Roy, 1992b). SULT2A1 expression and presence of DHEA and pregnenolone sulfate has been detected in the rat brain (Corpechot et al., 1981; Corpechot et al., 1983) where it is postulated they act as excitatory neurosteroids by being potent antagonists of the $GABA_A$ receptor (Paul and Purdy, 1992). DHEA sulfate is the most abundant steroid in the

human circulation and is known to play an important role in pregnancy and fetal development. During the third trimester of gestation, high levels of estrogen are reached (Katz and Kappas, 1967). This is derived from DHEA sulfate produced in the fetal adrenal gland due to the high level of SULT2A1 expression (Siiteri, 1966). Postnatally, the levels of DHEA sulfate drop only to rise again just before puberty. Thus SULT2A1 is expressed in an age dependent manner in the human adrenal (Hornsby, 1995; Suzuki et al., 2000). DHEA sulfate is also an important precursor for estrogen synthesis in postmenopausal women.

1.6.6 SULT2B

SULT2B1 localizes to chromosome 19q13.3, contains alternative exon and encodes two mRNAs. cDNAs for SULT2B1a and SULT2B1b encode proteins that are 350 and 365 amino acid respectively (Her et al., 1998). Both the enzymes have very narrow substrate preferences in comparison to SULT2A1. SULT2B1a extensively metabolises pregnenolone followed by DHEA and cholesterol that it weakly metabolises. Hence it is also known as pregnenolone sulfotransferase. SULT2B1b on the other hand strongly metabolises cholesterol and is thus known as cholesterol sulfotransferase. It metabolises pregnenolone also but with $1/10^{th}$ efficiency with which it sulfates cholesterol. Both SULT2B1a and SULT2B1b metabolise DHEA; however their affinity for the compound is several orders of magnitude lower than SULT2A1 (Javitt et al., 2001). Unlike SULT2A1, SULT2B1 does not sulfate bile acids, androsterone or estradiol. SULT2B1b is more widely expressed than SULT2B1a. SULT2B1a is expressed in the placenta, prostate and skin. Expression of SULT2B1b is not only predominant in the placenta, prostate and the skin but also extends to colon, lung, kidney, stomach, GI tract and thyroid (Geese and Raftogianis, 2001). Cholesterol sulfate aids keratinocyte differentiation

and is known to play a role in skin development and creation of the epidermal barrier (Kawabe et al., 1998; Shimizu et al., 2003).

1.6.7 SULT4 family

SULT4A1 was first identified in rat and human brain (Blanchard et al., 2004). *SULT4A1* is located on chromosome 22 (22q13.2-q13.31 and encodes a 33KDa protein (Falany et al., 2000). It is expressed only in the brain and no natural or xenobiotic substrates for this enzyme have yet been identified (Falany et al., 2000). The PAPS binding pocket of this enzyme is predicted to be smaller than other SULTs and hence might not accommodate the sulfuryl donor, PAPS. Allali-Hassani *et al* were also unable to demonstrate the binding of this enzyme to PAP. This could therefore mean that SULT4A1 is an atypical sulfotransferase that lacks the ability to use PAPS as a sulfuryl donor to carry out sulfation. (Allali-Hassani et al., 2007) However, predominant brain expression, and high degree of sequence identity across species are suggestive of an important physiological function which might take application of knock out or transgenic technologies to completely understand.

1.6.8 SULT6 family

SULT6A1 has been reported to be cloned from chicken liver and differentially down regulated in GH receptor deficient dwarf chickens. Recombinant Chicken SULT6A1 catalysed the sulfation of estradiol and corticosterone. SULT6B1 was discovered in human testis (Cao et al., 1999). However no substrates for human SULT6B1 have been found yet.

Bovine SULT (gene/cDNA) identified	Expression in bovine tissues	Commonly known endogenous substrates in human	Commonly known xenobiotic substrates in humans	References for expression in bovine tissue
SULT1A1	Lung and intestine	Iodothyronines, estrogens	Paracetamol, minoxidil, small phenolic compounds (4-nitrophenol)	(Baranczyk-Kuzma and Szymczyk 1987)
SULT1B1	Not known	Iodothyronines	Simple phenols (1-naphthol)	NA
SULT1C	Not known	Not known	4-nitrophenol, N-hydroxy-2-AAF	NA
SULT1E1	Placenta, epididymis, adrenal gland, fetal liver, skin, testis	Estrogens	Various estrogens: 2-hydroxyestrone, 2-hydroxyestradiol	(Nash, Glenn et al. 1988; Frenette, Leclerc et al. 2009; Lopparelli, Zancanella et al. 2010; Khatri, Frenette et al. 2011)
SULT2A1	Not known	Steroids: DHEA	Carcinogens: 1-hydroxymethylpyrene, hycanthone	NA
SULT2B1	Not known	DHEA, pregnenolone, cholesterol	Not known	NA
SULT4A1	Not known	Not known	Not known	NA

Table 1: Summary of major SULT genes identified in cattle (Zimin et al., 2009)

The expression of SULT genes in bovine tissues (see references column) and the commonly known substrates for assessing the enzyme activity in vitro (Gamage et al., 2006). NA stands for not applicable

1.7 Structure and function of sulfotransferases

Despite having broad and overlapping substrate specificities, individual SULT enzymes display high selectivity towards certain substrates. Generation of crystal structures of SULTs has helped understand the structural basis behind the substrate specificity which is crucial to elucidate the catalytic mechanism and function of SULTs. The majority of the structures solved to date are those bound with the co-factor PAP. Very few structures have been solved complexed with both cofactor PAP, or indeed PAPS, and substrate. This has hindered the elucidation of structural principles underlying the recognition and utilization of a given substrate (Gamage et al., 2006).

Mouse SULT1E1 was the first cytosolic sulfotransferase to be structurally characterised in the presence of PAP and the substrate 17β-estradiol (Kakuta et al., 1997). This was followed by structural characterisation of the first human sulfotransferase, SULT1A3 (Dajani et al., 1999a). Shown in figure 5 is the crystal structure of human SULT1A1 in the presence of the co-factor PAP and 4-nitrophenol.

1.7.1 PAPS binding region of SULTs

The study of SULT crystal structures has revealed that SULTs are generally globular proteins with a single α/β domain that forms a five stranded parallel β sheet surrounded by α helices (Gamage et al., 2006) and a three loop region that is often very disordered in the absence of PAPS and/or substrate (Allali-Hassani et al., 2007). The active site of SULTs is in a long groove at the end of the β sheet and it contains both the co-factor and the substrate binding site. Three amino acid residues namely Thr228, Arg258 and Gly260 (SULT1A1 numbering) are absolutely conserved in the PAPS binding region and are thought to play role in

PAPS binding (Allali-Hassani et al., 2007). Another motif that is highly conserved in SULTs is the 5'-phosphosulfate (PSB) binding loop which comprises 9 amino acid residues 45-TYPKSGTTW-54 (Kakuta et al., 1997) (Figure 6). The lysine residue within this motif (Lys 48) is believed to act as a catalytic acid in the reaction by protonating the phosphate-sulfate bridge oxygen of PAPS, thereby enhancing dissociation of the leaving group (Kakuta et al., 1998). The PSB loop is also thought to be important for orienting PAPS for sulfuryl transfer on to the acceptor substrate (Kakuta et al., 1998). The 3' phosphate region of PAPS interacts with residues from different parts of the polypeptide chain. These include Arg130, Ser138, Arg257, Lys258 and Gly259. The latter 3 residues are part of a highly conserved 257-RKGxxGxWK-265 motif. The catalytic centre of the enzyme is a histidine residue His108 that acts as a catalytic base abstracting a proton from the hydroxyl group on the acceptor substrate leaving behind a neucleophilic oxygen that 'attacks' the sulfur on PAPS. Mutating this residue abolishes enzyme activity (Kakuta et al., 1998). Recently Allali-Hassani *et al* revealed an unrecognised structural role for PAPS. They suggested that PAPS might play a role in priming the disordered substrate binding loop for subsequent interaction with potential substrates. This conclusion came from the finding that some substrates such as dopamine and 1-naphthol bound SULT1A3 and SULT1B1 respectively only in the presence of PAPS. The vice versa was not true however. Moreover it appears that PAPS binding orients the substrate binding loop to allow for specific binding. Enzymes that bind substrates in the absence of PAPS did not show activity towards its substrates. This suggests that PAPS affects substrate binding (Allali-Hassani et al., 2007).

1.7.2 Dimerization of SULTs

With the exception of mouse SULT1E1, the majority of the cytosolic SULTs exist as dimers in solution (Kakuta et al., 1997). They are capable of forming both homodimers as well as heterodimers. Cytosolic SULTs generally exist as homodimers in solution. They contain a conserved dimerization motif consisting of 10 residues near the C terminus represented by the consensus sequence KXXXTVXXXE called the KTVE motif (Petrotchenko et al., 2001). Mutation in the central Thr and Val in this motif resulted in the monomerization of human SULT1E1 that is known to exist as a dimer in solution. Similarly, mouse Sult1E1 exists as a monomer in solution but the substitution of Thr and Val by Phe and Glu respectively resulted in the formation of a dimer in solution (Kakuta et al., 1997). This demonstrates the significance of the dimerization motif in cytosolic SULTs. However, the role of dimerization in SULT activity if any has not yet been elucidated.

1.7.3 Substrate specificity of SULTs

Members of the SULT family display broad substrate specificity and yet some isoforms can be characterised by having preference for a specific substrate. For example an enzyme such as SULT1A1 is able to sulfate a wide range of xenobiotics with high affinity and on the other hand enzymes such as SULT1E1, SULT2A1 and SULT1A3 are highly specific for 17β-estradiol, DHEA and catecholamines respectively(Coughtrie, 2002). Thus in contrast to the PAPS binding site which is highly conserved, the substrate binding pocket exhibits a great deal of variety across SULTs (Gamage et al., 2006). It is important to determine what controls substrate specificity from a number of reasons including being able to predict the metabolic fate of drugs metabolised by sulfation and to enable the design of

selective inhibitors (Coughtrie, 2002). Analysis of crystal structures solved thus far has revealed that sequence identity at the amino acid level is definitely not an indicator of structural similarity or substrate specificity exhibited by the enzyme. A fine example of this is SULT1A1 and SULT1A3. Both the enzymes share 93% sequence identity and yet exhibit distinct substrate preferences. SULT1A1 prefers small uncharged phenolic compounds such as 4-nitrophenol, 1-naphthol and *p*-cresol whereas SULT1A3 prefers positively charged substrates such as dopamine and tyrosine (Brix et al., 1999; Dajani et al., 1998). The crystal structure of SULT1A1 was solved complexed with both PAP and 4-nitrophenol (Gamage et al., 2003). It was shown that the substrate binding site of the enzyme was very hydrophobic and plastic allowing the enzyme to adopt varying conformations so that it can interact with small aromatics (4-nitrophenol), L-shaped aromatics (diiodothyronines) and fused ring compounds such as 17β-estradiol (Gamage et al., 2003). On the other hand, the SULT1A3 structure was solved complexed with PAP. The structure was largely disordered mainly due to the absence of the bound substrate. The substrate binding site mostly contained acidic residues which favoured the binding of positive substrates (Dajani et al., 1999a). This explains the specificity of SULT1A3 towards positively charged residues such as dopamine. Mutation of amino acid Glu146 to its corresponding Ala residue in SULT1A1 resulted in SULT1A3 displaying SULT1A1 like enzyme properties (Dajani et al., 1998). Clearly Glu146 is a critical residue involved in determining the substrate specificity of SULT1A3 towards dopamine (Dajani et al., 1998). Another major study on mouse SULT1E1 explains its selectivity for 17β-estradiol over DHEA. It was proposed that residues in the substrate binding site of mouse SULT1E1 demonstrate a gating phenomenon whereby residues such as Tyr81 and Phe142 form a narrow gate that allows the entry of small endogenous compounds like

17β-estradiol but prevents the entry of DHEA which has a large 19-methyl group. Phe142 is conserved in all SULTs but Tyr81 is specific to mouse SULT1E1 and is responsible for regulating the 'gating' phenomenon (Petrotchenko et al., 1999).

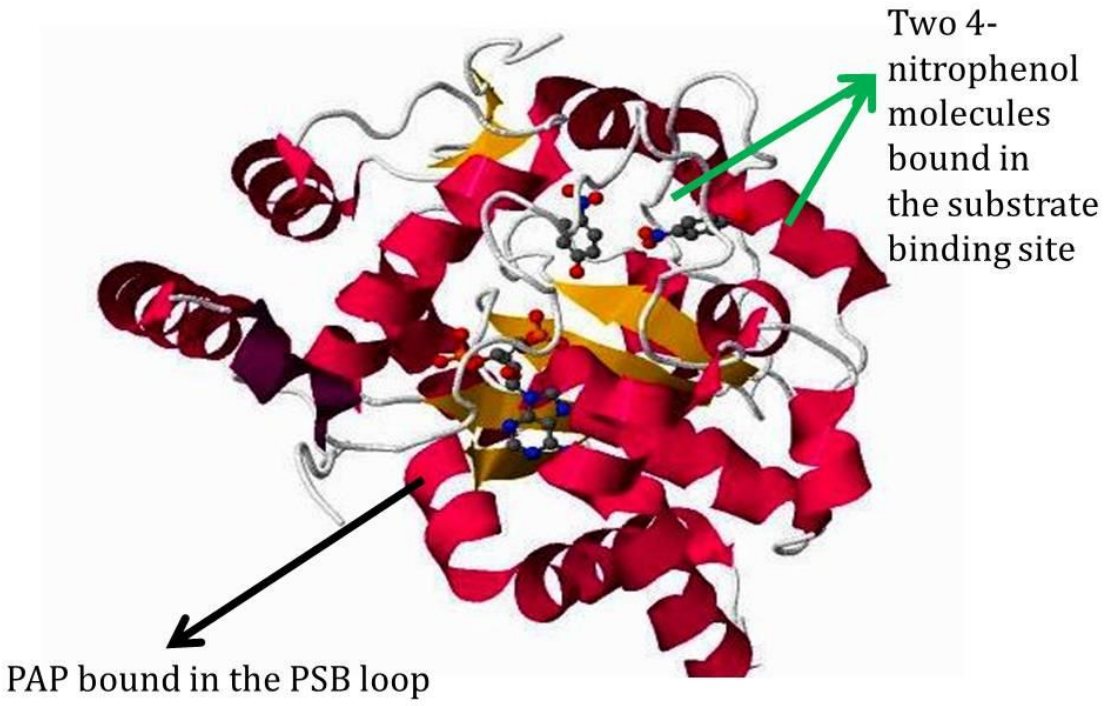

Figure 5: Structure of human SULT1A1.

The co-ordinates for human SULT1A1 crystallised in the presence of one molecule of PAP and two molecules of 4-nitrophenol were obtained from the protein data bank (http://www.rcsb.org/pdb/, entry 1LS6) A representation of the structure of human SULT1A1 is shown with α-helices displayed as red ribbons and β-sheets as golden yellow arrows (Gamage et al., 2003)

(Figure 6: figure legend on next page)

Figure 6: Sequence alignment of major bovine SULT isoforms

The regions highlighted are the ones that have important structural features. The yellow region corresponds to the highly conserved phosphate binding loop (PSB). It is involved in the binding of PAPS to the enzyme. The highlighted lysine acts as a catalytic acid in the reaction by enhancing dissociation of the leaving group. The histidine residue highlighted in green is an important catalytic residue in the active site of all SULTs. The turquoise region is the KXXXTVXXXE motif that plays a role in dimerization of SULTs. The PSB loop and the dimerization motif are fairly conserved in all SULTs except bovine SULT6B1.

1.8 Role of sulfotransferases in bioactivation and toxicology

Sulfation is predominantly recognised as a detoxification pathway where conjugating a sulfate moiety on to an endogenous or a xenobiotic compound increases its water solubility thereby facilitating excretion through bile and urine. However, bioactivation of dietary compounds, environmental pollutants, procarcinogens and therapeutic drugs by members of the sulfotransferase family to generate toxic metabolites has also been widely investigated.

Chemicals such as safrole and estragole which are alkoxy derivatives of allylbenzene are known to be potent rodent carcinogens. Hydroxyl metabolites of these compounds are more potent and readily sulfated compared to the parent compound (Wakazono et al., 1998). Brachymorphic mice which have genetic mutation that reduces their ability to synthesise PAPS (Sugahara and Schwartz, 1979) and hence the ability to carry out sulfation were resistant to tumour formation when fed with 1'-hydroxysafrole, the hydroxyl metabolite of the chemical safrole (Boberg et al., 1983). Mice treated with a sulfotransferase inhibitor named pentachlorophenol before being administered 1'-hydroxylsafrole showed a significant reduction in formation of DNA adducts and tumours (Boberg et al., 1983). *In vitro* experiments carried out by Glatt *et al* have shown that human purified and liver cytosol sulfotransferases sulfate and bioactivate a variety of

procarcinogens such as *N*-hydroxyarylamine, *N*-hydroxy-heterocyclic amines and arylhydroxamic acids (Gilissen et al., 1994a; Gilissen et al., 1994b; Meerman et al., 1994). SULT1A1 is responsible for the preferential metabolism and subsequent bioactivation of the dietary compound *N*-OH-PhIP (Ozawa et al., 1994) whereas expressed human SULT2A1 preferentially sulfates benzylic alcohols of polycyclic aromatic hydrocarbons (Glatt et al., 1995). The above examples provide evidence for the role of sulfation in the bioactivation of carcinogens.

Polyhalogenated aromatic hydrocarbons (PHAHs) such as polychlorinated biphenyls (PCBs), polychlorinated dibenzfurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) are environmental pollutants that have strong endocrine disrupting effects such as developmental and reproductive abnormalities(Neubert, 1997) and endocrine associated cancers such as breast cancer in animals and humans (Dorgan et al., 1999; Safe, 1995). They exert their endocrine disrupting effects in an estrogen receptor independent manner by inhibiting the activity of estrogen sulfotransferase, SULT1E1 (Kester et al., 2000; Kester et al., 2002). Indeed PHAHs are potent inhibitors of SULT1E1 and there is a possibility that they bring about endocrine disruption by regulating the bioavailability of 17β-estradiol. SULT1E1 is also known to sulfate iodothyronines (Kester et al., 1999b). Compromised thyroid function due to the effect of hydroxylated PHAHs during fetal development could possibly be explained by the inhibition of SULT1E1 by PHAHs (Darras, 2008).

In addition to procarcinogens, dietary compounds and environmental pollutants, therapeutic drugs are also bioactivated by sulfation. A classic example is the antihypertensive drug minoxidil (Campese, 1981). Minoxidil is predominantly sulfated by SULT1A1 (Falany and Kerl, 1990) and it is the sulfated metabolite that

mediates the pharmacological actions of minoxidil such as smooth muscle relaxation and vasodilation (Meisheri et al., 1993).

1.9 Sulfation of drugs

Drugs containing hydroxyl or amine groups are very often sulfated by sulfotransferases. Although various SULT isoforms are involved in drug metabolism, SULT1A1 and 1A3 are considered to be the major enzymes responsible for sulfating drug compounds. SULT isoforms 1A1, 1E1 and 1B1 are involved in the sulfation of local anaesthetics such as lidocaine and ropivacaine (Falany et al., 1999). SULT1A1 is primarily responsible for the sulfation of the widely used painkiller paracetamol (Reiter and Weinshilboum, 1982) and it is reported that 35% of the original dose of paracetamol was excreted in the urine in humans. Salbutamol, a bronchodilator used in the treatment of asthma, is mainly eliminated by sulfation. In a study carried out by Morgan, DJ *et al,* 1986 (Morgan et al., 1986), it was found that plasma levels of salbutamol sulfate were higher than salbutamol itself. Budesonide is a synthetic glucocorticoid used in the treatment of asthma and allergy. It is predominantly sulfated by SULT2A1(Meloche et al., 2002). This sulfation is all the more significant in budesonide metabolism when CYP3A predominantly responsible for its metabolism is inhibited by medical elements or dietary factors (Ameer and Weintraub, 1997).

It has been shown that sulfation of drugs possessing a chiral atom is stereoselective. This stereoselectivity could be due to different rates of sulfation or different affinity of sulfotransferases towards the two enantiomers. Terbutaline is a β-adrenoreceptor agonist is used in the treatment of lung obstruction disease. Pharmacokinetic studies have shown that (-)-terbutaline has a higher oral bioavailability than (+)-terbutaline (Borgstrom et al., 1989). In addition to this,

the sulfation rate of (+)- terabutaline in human liver is much higher than that of (-)-terbutaline (Walle and Walle, 1990). Salbutamol is a β-adrenoreceptor agonist whose target organ is the lung. SULT1A3 showed stereoselectivity in the affinity towards the two salbutamol enantiomers (Walle et al., 1993). In human lung cytosol, sulfation of (+) - salbutamol had a lower Km as compared to (-) - salbutamol. The V_{max} for both these enantiomers was approximately similar (Pacifici et al., 1996). Very complex enzyme kinetics was observed for 4-hydroxypropanolol (4-OHP) *in vitro* using human liver cytosol. 4-OHP, a metabolite of propanolol is a blocker of the β-adrenoreceptor. At low concentrations of the substrate (3μM) (Walle and Walle, 1991), stereoselectivity was observed with (+)- 4-OHP. However, no stereoselectivity was observed at the higher substrate concentration of 500μM. This led the authors to conclude that sulfation of 4-OHP at their respective concentration must be catalysed by different enzymes (Walle and Walle, 1991).

As with other drug metabolizing enzymes, SULTs too can be inhibited by chemicals, drugs or dietary factors. Inhibition of SULTs can lead to the build-up of toxic phase I metabolites resulting in adverse drug reactions. Non-steroidal anti-inflammatory drugs, mefenamic acid and salicylic acid are found to be potent inhibitors of SULT1A1 and 1A3 in human liver (Vietri et al., 2000). Flavonoids are chemicals present in fruit and vegetables. Quercetin , a natural flavonoid is a potent inhibitor of SULT1A1 activity in human liver (Walle et al., 1995). It affects the sulfation rates of salbutamol, paracetamol and minoxidil in human liver and duodenum (Eaton et al., 1996).

1.10 Interspecies differences in SULT expression and activity.

Interspecies differences have been observed in many enzymes responsible for drug metabolism and sulfotransferases are no exception. Differential expression of SULTs with respect to sex and tissue has been reported. Likewise, depending on species they belong to SULTs can have varying affinity for their substrates. One of the major differences is that in species other than humans, SULT expression is highly sexually dimorphic or sex related. SULT1A1 content in adult male rat liver is 1.5 times higher than the female rat liver whereas on the other hand, expression of mouse SULT1A1 was about twice as high in females as compared to the males (Honma et al., 2001). SULT2A1 is expressed more in adult female rats as compared to male rats. Expression of SULT2A1 in rats is regulated by growth hormone and sex related differences in rats was observed due to differential expression of pituitary growth hormone between female and male rats (Ueda et al., 1997). Rat Sult1E1 is specifically expressed only in the liver cytosol of young adult males. It was found to be absent in prepubertal males (27days), senescent males (800 days) and in female liver cytosols (Demyan et al., 1992). Several studies have shown that mRNA levels of Sult1C1 were higher in male rats as compared to females (Klaassen et al., 1998; Liu and Klaassen, 1996). N-OH-2AAF, a carcinogen is metabolized to its sulfo conjugate and induces tumour formation in rats. However, due to a gender difference in Sult1c1 expression, tumours develop more rapidly in male mice as compared to females (DeBaun et al., 1970). In humans, SULT1C1 are expressed in kidney, liver, ovary, stomach and colorectum (Dooley et al., 2000). However, in rats Sult1c1 is mainly expressed in liver with little or no expression detected in kidney, lungs, intestine, spleen, brain or heart (Dunn and Klaassen, 1998). Sex related differences were observed not only in the

levels of expression but also in the sites of expression. Male dog expresses SULT1D1 in liver, jejunum, ileum, and colon and to a lesser extent in duodenum whereas the female dog expresses SULT1D1 to a higher extent in liver and duodenum but to a lesser extent in ileum and jejunum. SULT1D1 has so far been identified in rat, mouse and dogs but not humans (Oddy et al., 1997). Differences also exist in tissue distribution and abundance of SULT2B1 in rats and mice from that of human isoforms. High levels of SULT2B1b were detected in skin and intestine of all 3 species. In rats little or no SULT2B1a expression was detected in tissues except brain and testis in which SULT2B1a is expressed but not 2B1b (Kohjitani et al., 2006). In mice SULT2B1a message is highly expressed in brain and spinal cord whereas no SULT2B1 message or protein has been detected in human brain (Shimizu et al., 2003). To date SULT2B1 protein expression in mice or rat brain tissue has not been reported. SULT2B1b is predominantly expressed in human placenta and prostrate whereas SULT2B1b is barely expressed in the rat placenta and not in the prostrate (Kohjitani et al., 2006). In addition to this differences have also been observed in the SULT isoforms identified. For example, various members of the SULT1A family (SULT1A1, SULT1A2, SULT1A3, and SULT1A4) have been identified in humans as opposed to only a solitary SULT1A1 being identified in other species. SULT1A3 exists only in humans and is known to sulfate endogenous compounds such as dopamine, norepinephrine and tyrosine. Conversely all other SULT1A members have very low affinity towards these chemicals suggesting a human specific role for SULT1A3 in the metabolism of catecholamine neurotransmitters. Rats have 4 members of the SULT2A family whereas only one member of this family (SULT2A1) has been identified in humans so far. No human equivalents of Sult5a1, Sult3a1 in mouse and the Sult6a1 gene in chicken have yet been identified (Gamage et al., 2006). Differences in the ability to

sulfate various endogenous and xenobiotic substrates also exist between humans and other species. Rat Sult1A1 is inactive for the sulfation of iodothyronines whereas human SULT1A1 is capable of catalysing iodothyronine sulfation. Canine and rat Sult1A1 have activity against dopamine which is in contrast to human SULT1A1 which has a very low affinity for dopamine. The SULT2B1a isoform in rats and humans sulfates cholesterol to a lesser extent as compared to Sult2B1a isoform in mice (Kohjitani et al., 2006)

1.11 Sulfotransferases in cattle and other livestock animals

In comparison to humans and laboratory animals, xenobiotic biotransformation has not been extensively studied in members of the livestock species. Majority of the research carried out on livestock and other veterinary animals is centred on cytochrome P450 mediated biotransformation of drugs. A few reviews focus on phase 2 drug metabolising enzymes such as UGTs, N-acetyltransferases and glutathione-S-transferases but the literature on sulfotransferase mediated drug and xenobiotic metabolism in livestock species is currently very limited. Bovine SULT1E1 was the first cDNA cloned as a known sulfotransferase. The protein was found to be 295 amino acids long and of approximately 34kDa molecular weight (Nash et al., 1988). Subsequently cDNA encoding phenol sulfotransferase (PST) was isolated from bovine lung. It was discovered that two forms of PST exist in the human lung. One which sulfates phenolic compounds such as 1-naphthol and p-nitrophenol (SULT1A1) and the other was specific towards monoamine substrates such as catecholamines (dopamine) (SULT1A3). Bovine lung was found to have only the thermostable form of PST, i.e SULT1A1 (Baranczyk-Kuzma and Szymczyk, 1987). A study carried out by Watkins and Klassen showed that cattle and sheep sulfotransferases had the highest activity towards 2-naphthol as the substrate

among a range of species tested (Watkins and Klaassen, 1986). Similiar observations were recorded by Sivapathasundaram *et al* 2003 where cytosolic SULT activity measured with 2-naphthol was significantly higher in cattle than in rat. Comparison of sulfation capacities of cattle, deer and rat showed that sulfation was similar in cattle and deer but differences were observed between the ruminants on one side and rat on the other (Sivapathasundaram et al., 2003).

1.12 The need for studying SULT mediated drug metabolism in cattle

For the elimination of a wide variety of chemicals, living organisms have evolved enzyme systems that bring about the biotransformation of these compounds to hydrophilic entities that can safely excreted from the body. This is termed as metabolism. To date numerous reviews have focussed on drug and xenobiotic metabolism in humans and laboratory animals. However, literature on metabolism in food producing animals/livestock species is very limited. Lack of comparative data on drug metabolism between food producing species results in the extra-label use of veterinary products labelled for use in other animal species. Due to the differences in expression and activities of drug metabolising enzymes, extrapolation of metabolism data between species is highly inaccurate. Extra label use of drugs between species without sufficient knowledge on drug metabolism in the target species could result in adverse drug reactions. Residues of drugs/xenobiotics can end up in edible tissues of food producing animals and reach humans through the food chain posing considerable risk to human health. This is of grave concern to the livestock and pharmaceutical industry. Authorities regulating the usage of veterinary drugs in livestock demand evidence for safe and effective use of these drugs prior to licensing and marketing of the drug. For all these reasons it becomes important to study drug metabolism in food producing

species. Sulfotransferases are the second most abundant phase 2 conjugating enzymes found in human liver and despite this very little research has been done on SULTs in food producing animals (Evans and Relling, 1999).

1.13 UDP glucuronosyltransferases

Like sulfation, glucuronidation also is a phase II conjugation reaction catalysed by a family of enzymes known as UDP glucuronosyltransferases (UGTs). UGTs are involved in the conjugation of glucuronic acid (UDPGA) onto lipophilic substrates, thereby increasing their water solubility and enhancing excretion through bile and urine. Glucuronidation reactions are generally detoxification reactions as conjugation of glucuronic acid group on a toxic substrate makes it relatively nontoxic (Tukey and Strassburg, 2000). However in some instances glucuronidation can also lead to the bioactivation of substrates. For example morphine-6-glucuronide is pharmacologically more active than its parent compound (Shimomura et al., 1971). The addition of the glucuronic acid moiety generally occurs at hydroxyl, amine, carboxyl, carbonyl and thiol groups. UGTs can conjugate a variety of endogenous substrates such as bilirubin, thyroxine, estradiol, testosterone, androsterone and hyodeoxycholic acid. In addition to this xenobiotic compounds serve as UGT substrates including drugs such as morphine, chloramphenicol, acetaminophen, propofol and non-steroidal anti-inflammatory drugs (NSAIDs). Environmental compounds such as plant derived dietary flavonoids and carcinogens such as *N*-OH-PhiP are also metabolised by UGTs (Tukey and Strassburg, 2000). Indeed about 35% of all drugs metabolized in phase II are biotransformed by UGTs (Evans and Relling, 1999). It is also involved in the direct clearance of 1 in 10 of the top 200 prescribed drugs in humans (Williams et al., 2004).

UGT enzymes are bound to the internal membrane of the endoplasmic reticulum (ER) and face the luminal side of the ER. UGTs possess a highly hydrophobic C terminus which is believed to anchor the protein to the cytoplasmic side of the ER. The N terminal region is not very well conserved and hence is thought to be involved in the binding of the substrate (Tephly and Burchell, 1990).

1.13.1 UGT1 family

The UGT gene superfamily is divided into 4 familes, UGT1, UGT2, UGT3 and UGT8 based on sequence identities at the amino acid level (Mackenzie et al., 2008). The *UGT1A* locus contains a unique first exon and four common exons (2-5) This gives rise to a common C-terminus encoded by the 4 common exons. The first exon unique to every gene of the *UGT1A* family encodes the variable N-terminus of the UGT1A family (Mackenzie et al., 2005). In humans, 13 unique first exons have been identified (UGT1A1-13) that encode 9 functional proteins (Gong et al., 2001). In rats, 9 first exons encoding 9 functional proteins have been identified (UGT1A1-9) (Emi et al., 1995) whereas in mice the *Ugt1a* subfamily contains 14 exons that code for 9 functional Ugt1a isoforms.

Human UGT1A transcripts are highly expressed in liver, kidney and GI tract (Fisher et al., 2001; Mojarrabi and Mackenzie, 1998; Strassburg et al., 1998). Similarly, mRNA expression of rat UGT1A isoforms has been detected predominantly in the liver and intestine whereas in mice several Ugt1a transcripts have been detected in liver, kidney, stomach, esophaegus, small intestine and colon (Emi et al., 1995; Shelby et al., 2003). UGT1A isoforms demonstrate high substrate specific redundancy in the glucuronidation of phenolic compounds. The simple phenols such as 4-nitrophenol, 1-naphthol, and 4-methylumbelliferone serve as excellent substrates for majority of the UGT1A family members except

UGT1A4. Complex and bulky phenol form excellent substrates for UGT1A6, 1A8 and 1A9. However, naturally occurring anthraquinones and flavonoids are not good substrates for UGT1A6. Glucuronidation of bilirubin is exclusively catalysed by UGT1A1. In addition to this primary amines form excellent substrates for UGT1A6 and UGT1A9 (Tukey and Strassburg, 2000).

1.13.2 UGT2 family

Unlike *UGT1* genes, *UGT2* genes are encoded by 6 individual exons and divided into 2 familes namely *UGT2A* and *UGT2B*. UGT2A1 mRNA is expressed in the nasal epithelia of humans and rats (Heydel et al., 2001; Jedlitschky et al., 1999). Gene duplication of *UGT2A1* in humans results in the formation of UGT2A2 which is expressed in liver. Their function is not entirely known (Tukey and Strassburg, 2001). Mice contain 3 *Ugt2a* gene duplication products that have resulted in the formation of *Ugt2a1*, *Ugt2a2*, *Ugt2a3*. Humans, rats and mice all contain seven unique *Ugt2b* genes (Mackenzie et al., 2005). UGT2B members are expressed not only in the liver and intestine but also in hormone responsive tissues such as testis, uterus, mammary gland and brain where they have been known to conjugate endogenous steroids (Shelby et al., 2003; Turgeon et al., 2001).

1.13.3 UGT3 Family

Unlike UGT1 and UGT2 familes that use UDP-glucuronic acid as a sugar donor, UGT3A1 was found to catalyse the transfer of *N*-acetylglucosamine from UDP-*N*-acetyl glucosamine to ursodeoxycholic acid. In addition to ursodeoxycholic acid, UGT3A1 also had activity towards 17α-estradiol and 17β-estradiol and the substrates of UGT1 and UGT2 families such as 1-naphthol and 4-nitrophenol. UGT3A1 was predominantly expressed in the liver and kidney with minor expression in the gastrointestinal tract (GI tract) tract as well (Mackenzie et al.,

2008). On the other hand, UGT3A2 transcripts have been detected in the thymus, testis and kidney but not in the liver or the GI tract. The absence of UGT3A2 in major drug metabolising tissues suggest that the enzyme might play a role in protecting organs against toxic elements rather than perform a traditional drug metabolism role. Like UGT3A1, UGT3A2 also does not prefer UDPGA as a sugar donor. UGT3A2 uses UDP glucose and UDP-xylose as sugar donors to glycosidate a wide range of substrates such as bioflavones, 1-hydroxypyrene, 4-methylumbelliferone and estrogens. Unlike UGT3A1, UGT3A2 does not accept UDP *N*-acetylglucosamine as a sugar donor (MacKenzie et al., 2011).

1.13.4 Species, tissue and gender differences in UGT expression and activity

Species, gender and tissue specific differences in expression and activity of UGTs have been documented. Analysis of UGT activity in hepatic homogenate preparations from rat, sheep, cattle and swine towards varied substrates such as 1-naphthol, 4-nitrophenol, morphine, diethylstilbesterol, bilirubin, estrone and testosterone revealed that sheep liver was the most efficient at glucuronidation of all the substrates except estrone where the highest activity was observed in cattle liver (Smith et al., 1984). Apart from bilirubin, UGT activity towards all the above mentioned substrates was higher in the livestock species than rat (Smith et al., 1984). Traditionally rodents such as rats and mice and non-primate species such as the dog have been used in toxicity testing of new chemical entities. Several studies have been carried out to address interspecies differences between dogs and humans with a view to make reasonable extrapolations of pharmacokinetic and toxicity data from dogs to humans. Ethinylestradiol glucuronidation, brought about mainly by UGT1A1 and UGT2B7 isoforms was twice as much in dogs as compared to humans (Sharer et al., 1995). No propofol glucuronidation was

detected in dog liver unlike the human liver. Stereo and regio-selective differences were seen in the glucuronidation of certain substrates such as oxazepam and morphine. In humans and dog the *S*-enantiomer was preferentially glucuronidated whereas in monkey it was the *R*-enantiomer of oxazepam that was preferred (Sisenwine et al., 1982). Human liver microsomes selectively metabolised morphine at the 3- and 6-positions whereas in dogs the 3 position of morphine was preferentially glucuronidated (Soars et al., 2001). Extra hepatic glucuronidation in dogs was poorer than in humans (Soars et al., 2001). Tissue specific differences were also observed in glucuronidation of 4-nitrophenol by UGT1A6 in mice and rats. The duodenum in rats was more efficient than the liver in 4-nitrophenol glucuronidation. Vice versa was noted for mouse liver (Shiratani et al., 2008). Gender differences in UGT expression and activity have been documented in rats. In Wistar rats, the rate of bilirubin glucuronidation was twice as much in females as compared to the males. Castration of males resulted in increased bilirubin glucuronidation (Muraca and Fevery, 1984). On the other hand ovariectamized female rats displayed a reduction in activity towards bilirubin (Muraca and Fevery, 1984). Expression of rat UGT2B3 was higher in males than in females which could be reduced by castration of males and restored by testosterone treatment (Buckley and Klaassen, 2007; Strasser et al., 1997).

1.14 Aims and hypothesis

This investigation was carried out to characterise SULT isoforms involved in the sulfation of xenobiotics and steroids in bovine liver. To date majority of the research in this field has been done on human sulfotransferase isoforms. Antibodies and probe substrates against major drug metabolising human SULT isoforms are available. There resources were used to study bovine SULT isoforms.

Given the high degree of sequence similarity between human and bovine SULTs (>70%), an assumption that bovine SULTs would be similar in activity to human SULTs was made. The following short aims were set out to accomplish the goal of the project and test the above mentioned hypothesis.

1. Using human SULT antibodies and probe substrates, expression and activity of SULT1A1, 1B1, 1E1 and 2A1 in bovine liver was established.
2. Recombinant bovine SULT1A1, 1B1, and 1E1 were cloned and expressed in *E. coli.* Their activity measured using human probe substrates was compared to recombinant human SULT isoforms.
3. Steroid metabolism brought about by SULTs and UGTs was studied using estradiol (a commonly used cattle growth enhancer) and model systems such as microsomes, S9, cytosol and hepatocytes.

2 Materials and Methods

2.1 Bovine livers

Given below are the details of bovine liver tissue used in this study

No of livers	Sex	Age	Breed	Treatment
8	Male	2yrs old	Angus	Untreated
8	Female	2yrs old	Angus	Untreated
4	Female	2yrs old	Angus	Treated with an exogenous progestin

Table 2: Information on bovine livers used in this study

2.1.1 Preparation of cytosol and microsomes

Big pieces of frozen liver (above) were received from Pfizer Animal health, Kalamazoo, Michigan, USA. On arrival they were immediately stored at -80°C until further use. Small pieces of frozen liver were obtained from the big piece using a hammer. The small piece obtained was then used in the preparation of cytosol and microsomes. Firstly, the small piece was weighed and resuspended in 20% (w/v) ice cold sucrose HEPES (*4- (2-hydroxyethyl)-1-piperazineethanesulfonic acid*) buffer (0.25M Sucrose, 10mM HEPES, 3mM 2-mercaptoethanol, pH 7.4). Once thawed, the tissue was chopped up into small pieces using a pair of scissors. The homogenate was transferred to a Teflon glass homogeniser and thoroughly homogenised using a mechanical homogenizer. The homogenate was then spun at 10,000g for 15 minutes at 4°C to pellet the cell debris and plasma membrane. The supernatant was then spun at 100,000g for 1 hour at 4°C in an ultracentrifuge (Kontron-Centrikon T-2070) to separate microsomes (pellet) and cytosol (supernatant). The microsomes were resuspended in ice cold sucrose HEPES buffer. 1ml buffer was used for every 1g wet weight of original tissue. Both

microsomes and cytosol were divided into aliquots, snap frozen in liquid nitrogen and stored at -80°C until required.

2.2 Radiolabelled enzyme assays for determination of bSULT activity

2.2.1 General

Radiolabelled assays were carried out to determine activity/kinetics of recombinant bovine SULTs and bovine liver cytosol against a battery of substrates. Radioisotopes such as sulfur 35 (^{35}S) and tritium (^{3}H) were used in this project. PAPS, the sulfuryl donor in sulfation reactions was labelled with ^{35}S whereas steroid substrates such as 17β-estradiol and pregnenolone were labelled with ^{3}H. Radiolabelled compounds were purchased from Perkin-Elmer NEN. Non radiolabelled PAPS (>99% purity) was obtained from Prof. H. Glatt (Institute for Human Nutrition, Potsdam, Germany). Enzyme kinetics assays carried out for every set of recombinant protein/ liver cytosol and a given substrate were optimised for protein concentration, incubation time, PAPS concentration, buffer and pH.

2.2.2 Radiolabelled ^{35}S PAPS assay

2.2.2.1 Theory behind the assay

The assay was developed by Folds and Meek in 1973 (Foldes, 1973). SULTs catalyse the transfer of the sulfuryl group from the co factor PAPS on to the substrate molecule in a typical sulfation reaction. The radiolabelled sulfate is transferred from PAPS on to the substrate, thus making the substrate more water soluble. Any unreacted radiolabelled PAPS are precipitated by the addition of barium acetate, barium hydroxide and zinc sulfate in a sequential manner. The substrate that has now become water soluble due to the addition of the sulfuryl

group remains in the aqueous solution. Radiolabelled product is then counted on a scintillation counter. Given below is an illustration of this assay

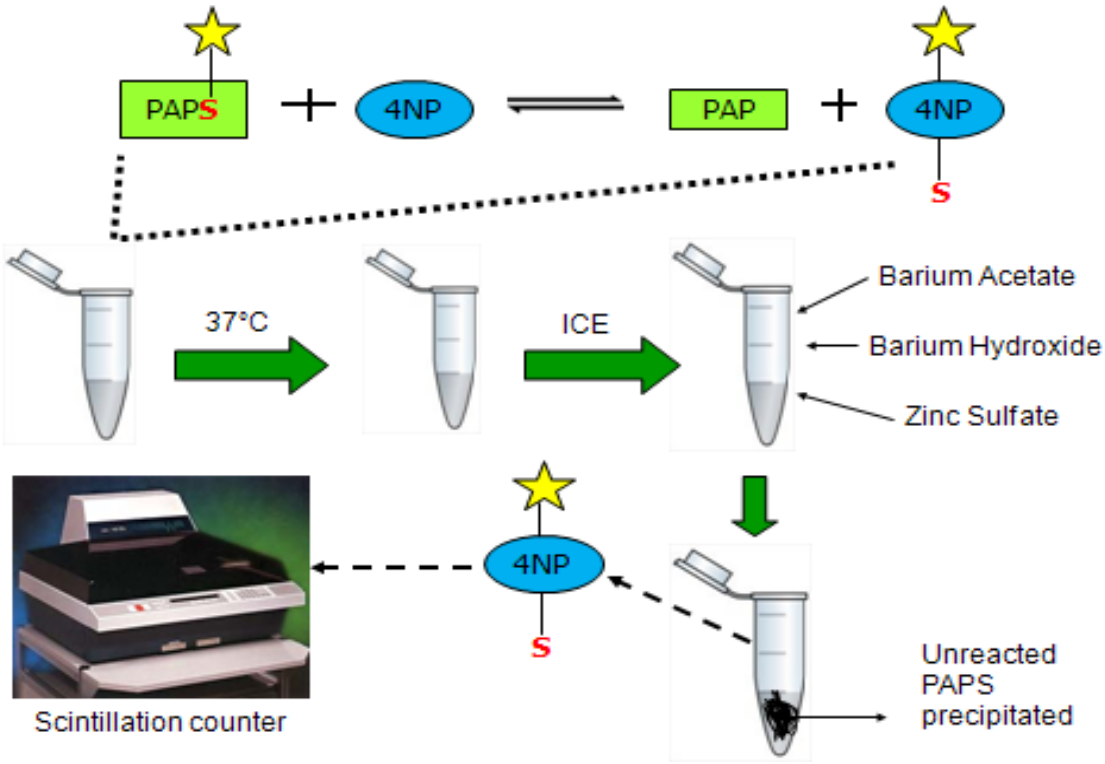

Figure 7: A schematic diagram of the ^{35}S PAPS based assay.

The yellow star represents radioactivity. The reaction was carried out at 37°C and stopped by placing the tubes on ice. Supernatant containing soluble product is read on the scintillation counter.

2.2.2.2 Performing the assay

10-20µl of [^{35}S] PAPS was diluted appropriately (usually made up to 1 or 1.5ml with water) such that the diluted stock produces counts per minute (cpm) in the 100,000-150,000 range. 10µl of this diluted stock were added to each reaction in addition to the non-radioactive PAPS.

The reaction mixture was prepared in a total volume of 160µl containing 0.1M phosphate buffer, substrate, protein and PAPS. [^{35}S] PAPS was prepared in a 1:1 ratio with 20µM non radiolabelled PAPS. This was done because of the reduced

sensitivity of the scintillation counter at high amounts of radioactivity. The reactants were assembled as follows.

Reactants	Volume (µl)
0.1M KPO_4 buffer (pH various)	100
Substrate (various)	20
Protein (various)	20
Hot and Cold PAPS (various)	20
Total	**160**

Table 3: Reactants assembled in a typical ^{35}S PAPS based assay.

Reactions were started by the addition of PAPS and incubated at 37°C for 15-30 minutes. Blank tubes containing everything but the substrate were set up to monitor the precipitation of the unreacted radiolabelled PAPS. Reactions were stopped and quenched by placing the tubes on ice and addition of 200µl of 0.1M barium acetate, barium hydroxide and zinc sulfate in the given sequence. The samples were then mixed and centrifuged at 16,000g for 4 minutes in order to precipitate any unreacted PAPS. 500µl of the resulting supernatant were added to 4 ml scintillation fluid (Emulsifier safe, Perkin Elmer) and counted at 1 minute per vial on a scintillation counter (Beckman Coulter, High Wycombe, UK).

2.2.3 Radiolabelled ^{3}H solvent extraction assay

2.2.3.1 Theory behind the assay

As opposed to the [^{35}S] PAPS assay where the cofactor PAPS was radiolabelled, the substrate was radiolabelled in the [^{3}H] based assay. Steroid substrates used were

insoluble in aqueous solutions and hence cannot be precipitated easily. For this reason another assay was used which instead of precipitating the unreacted radioactivity separates it in the organic solvent while the product is partitioned in the aqueous phase. As the sulfation reaction proceeds, the sulfuryl group from PAPS is transferred onto the substrate, thus making the substrate more water soluble than before. Addition of chloroform separates the unreacted radiolabelled steroid in the organic solvent while the radiolabelled product remains in the aqueous phase. It is this radiolabelled product that is counted on the scintillation counter. Given below is an illustration of the solvent extraction assay.

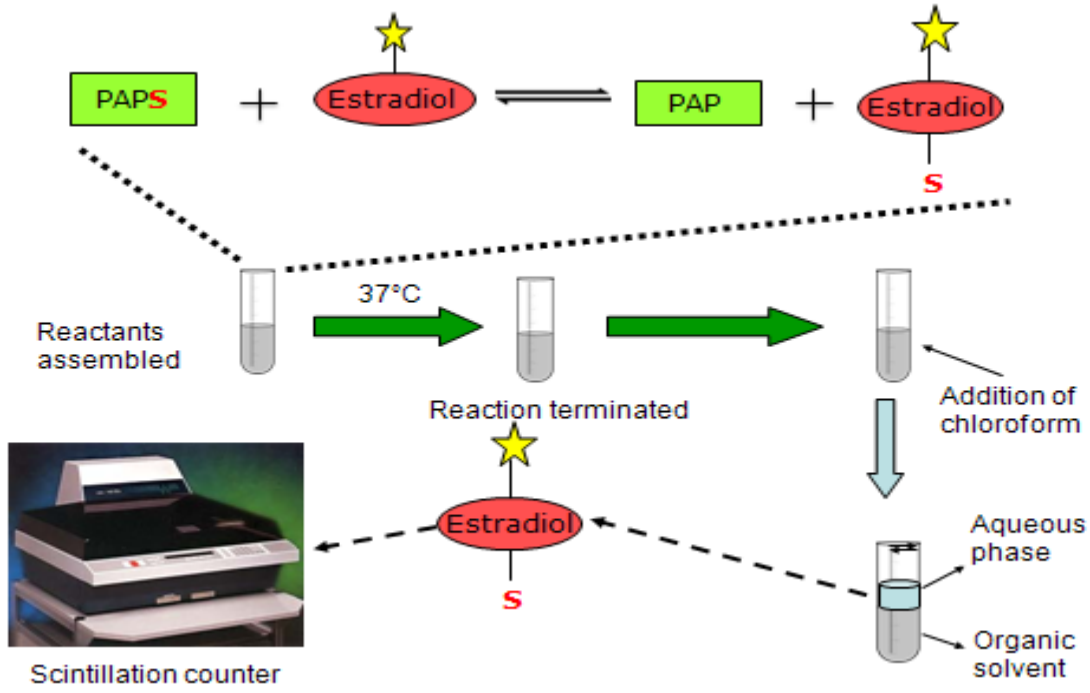

Figure 8: A schematic diagram of the [^{3}H] based assay.

The yellow star represents radioactivity. The reaction was carried out at 37°C and stopped by the addition of chloroform. Aqueous phase containing the soluble product is read on the scintillation counter.

2.2.3.2 Performing the assay

10-20µl of [^{3}H] substrate was diluted appropriately such that the diluted stock produces cpm in the 100,000-150,000 range. 10µl of this diluted stock were added to each reaction in addition to the non-radiolabelled substrate.

Reaction mixtures were prepared in a total volume of 200µl containing 0.1M phosphate buffer, [^{3}H] substrate, protein and PAPS. The reactants were assembled as follows

Reactants	**Volume (µl)**
0.1M KPO_4 buffer (pH various)	100
[^{3}H] Substrate	10
Non-radiolabelled substrate (various)	20
PAPS (various)	20
Protein (various)	20
dH_2O	30
Total	**200**

Table 4: Reactants assembled in a typical ^{3}H based assay.

Reactions were started by the addition of PAPS and incubated at 37°C for 15-30 minutes. Blank tubes containing everything but the PAPS were set up to monitor the extraction of radiolabelled substrate. Reactions were stopped and quenched by adding 300µl ice cold water and 3ml chloroform to each tube. The samples were then mixed and centrifuged at 700g for 4 minutes in order to separate any unreacted substrate in the chloroform while the radiolabelled product remains in the aqueous phase. 200µl of the aqueous solution was added to 4ml scintillation

fluid (Emulsifier safe, Perkin Elmer) and counted at 1 minute per vial on a scintillation counter (Beckman Coulter, High Wycombe, UK).

2.2.4 Analysis of data obtained from radioactive assays

Counts obtained from radioactive assays were used for activity and specific activity calculations. Radioactive counts were entered in an Excel spreadsheet and the equation given below was used for the calculation of specific activity.

$$\frac{\text{(C-B/TCPM) x ratio count correction x Total PAPS/Substrate}}{\text{Protein x Incubation time}}$$

Test Counts	C
Blanks	B
Test count - Blank	C-B
Total count per minute	TCPM

Total count per minute is the number of counts generated by the radioactive component in an assay before the reaction is allowed to proceed.

500µl out of the total 760µl (160µl reaction mixture plus 600µl of the total stop solution) were counted on the scintillation counter in the [^{35}S] PAPS assay. Hence the ratio count correction for this assay is 760/500 = 1.52

Similarly, 200µl of the total 500µl (200µl reaction mixture plus 300µl ice cold water) were counted on the scintillation counter in the solvent extraction assay. Hence the ratio count correction for this assay is 200/500 = 2.5

Total PAPS/ Substrate (pmol) is the total amount of labelled and unlabelled substrate used in the reaction. This parameter takes into account the fact that both labelled and unlabelled PAPS/substrate are turned over in a reaction whereas only the labelled component of the assay is reflected in the counts. Amount of PAPS/substrate added to the reaction is known but the amount of radioactivity added to the reaction in 10µl is calculated using values given in the data sheet obtained with the radioactive isotope from the manufacturer.

For enzyme kinetics analysis, data was imported from the Excel spreadsheet into Graph Pad Prism software. GraphPad Prism was used to calculate V_{max} and K_m values using either the Michaelis-Menten equation or the partial substrate inhibition equation. The equations used are given below. K_i in the partial substrate inhibition equation stands for the inhibition constant.

Michaelis-Menten equation	**Partial substrate inhibition**
$\mathbf{Y= V_{max} X/ (K_m +X)}$	$\mathbf{Y=V_{max} X/(K_m+X(1+X/K_i))}$

2.2.5 Radiolabelled ^{35}S PAPS assays: List of substrates used in the substrate screen assays and the solvent used for dissolving them

Substrate	Solvent
1-naphthol	3% DMSO plus dH_2O
2-aminophenol	dH_2O
2-naphthol	3% DMSO plus dH_2O
2-nitrophenol	dH_2O
2-phenylphenol	3% Ethanol plus dH_2O
3-nitrophenol	dH_2O
4-amylphenol	100% Ethanol
4-isopropylcatechol	dH_2O
4-isopropylphenol	dH_2O
4-nitrophenol	dH_2O
4-phenylazophenol	100% Ethanol
17β-estradiol	1,2 Propanediol
Apomorphine	dH_2O
Dobutamine	dH_2O
Paracetamol	dH_2O
Pregnenolone	1,2 Propanediol
Sesamol	dH_2O

Table 5: Substrates used in substrate specificity profiling assays

List of substrates used and the corresponding solvents that were required to dissolve them. dH_2O refers to distilled water and DMSO stands for dimethyl sulfoxide.

2.3 Sodium dodecyl sulfate (SDS)-PAGE gel electrophoresis and immunoblotting of recombinant bSULTs (and in liver cytosol)

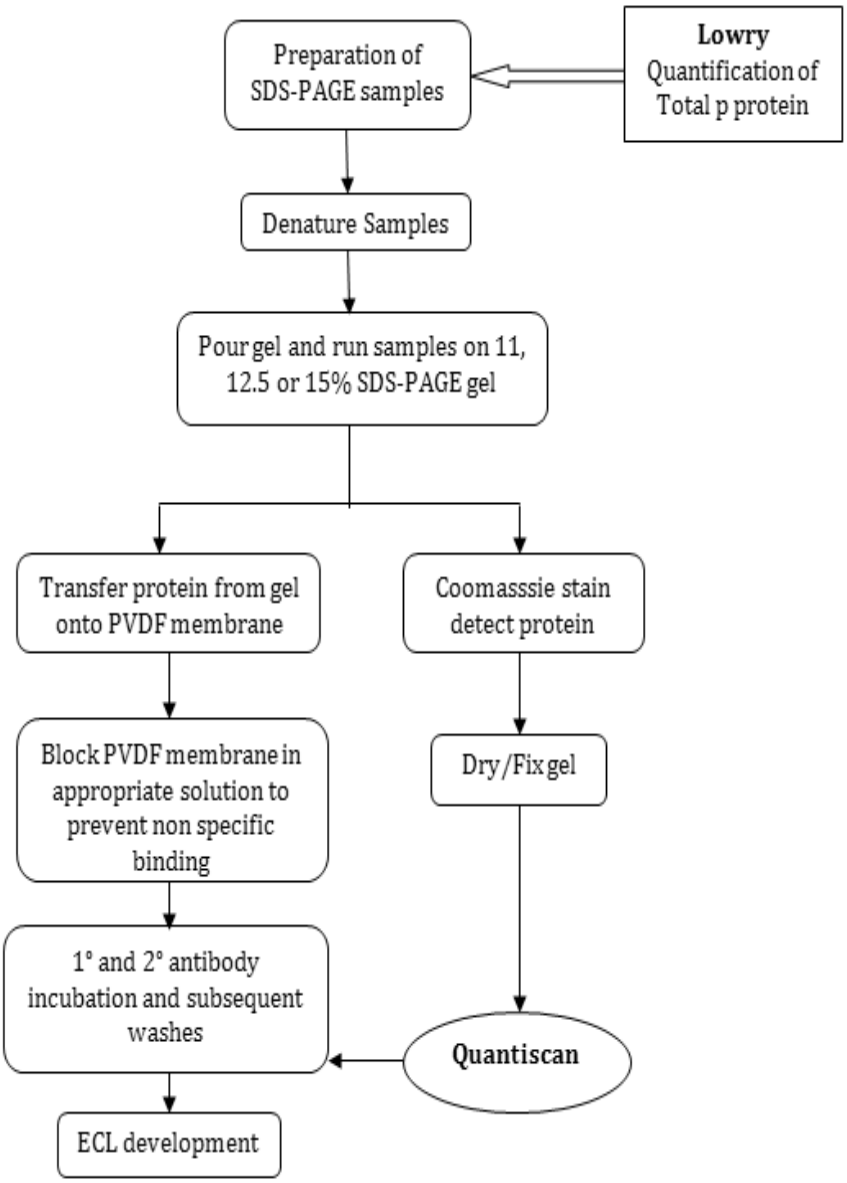

Figure 9: Overview of immunoblotting

2.3.1 Purpose

- Detect the presence of purified recombinant bovine SULT and of recombinant SULTs in cell free/crude extract.
- Determine SULT expression in bovine liver cytosol

2.3.2 Lowry assay for quantification of total protein

No	Reagents	Storage	BSA standards (mg/ml)
1	$CuSO_4.5H_2O$, 2% (w/v)	4°C	0.02
2	NaK tartrate, 2% (w/v)	4°C	0.05
3	Na_2CO_3 in 0.1M NaOH, 2% (w/v)	4°C	0.1
4	Reagent 4	Made fresh	0.15
5	Folin's Ciocalteu: dH_20 (1:1)	Made fresh	0.2

Table 6: Details of reagents used in Lowry assay

Reagent 4 was prepared by mixing reagents 1, 2 and 3 in the ratio of 1:1:100. 250µl of reagent 4 were added to duplicate 50µl aliquots of Bovine Serum Albumin (BSA) standards and samples that were diluted appropriately (1:50, 1:100, and 1:200). All the samples and standards were mixed in a vortex mixer and left to incubate at room temperature for 10 minutes. 25µl of reagent 5 was added to each tube and the tube was mixed immediately. After addition of reagent 5 the standards and samples were incubated at room temperature for minimum of 30 minutes. The OD_{600} was read on a plate reader (SpectraMax 190, Molecular

Devices) and the software (SoftMax Pro 2.6.1) was used to generate a standard curve and derive the sample protein concentration from the standard curve.

2.3.3 Pouring and running SDS-PAGE gel

2.3.3.1 Small format gels

The maximum number of samples that could be loaded on these gels was 15. 15% SDS-PAGE gel was prepared as follows. TEMED stands for NN'N'-tetramethylethylenediamine and in combination with ammonium persulfate (APS) helps in the cross linking of acrylamide.

Reagents (ml)	**Details**	**Separating gel volume (ml)**	**Stacking gel volume (ml)**
Water	None	3.5	4
Acrylamide	30% acrylamide, 0.8% bis	4	0.5
Lower Tris buffer	1.5M Tris-HCl, pH 8.8	0	0
Upper Tris buffer	0.5M Tris-HCl, pH 6.8	2.5	0.5
Ammonium persulfate	10%	0.1	0.07
TEMED	-	0.01	0.007

Table 7: Reagents required for SDS gel electrophoresis (small).

Samples to be loaded on gels were mixed with 2X loading buffer containing SDS (100mM Tris-HCl pH6.8, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 4% (w/v) SDS, 20% (v/v) mercaptoethanol) and incubated at 100°C for 5 minutes.

The gels were run at 200V in BioRad electrophoresis tanks filled with running buffer (25mM Tris, 191mM Glycine, 1% SDS).

2.3.3.2 Large format gels

The maximum number of samples that could be loaded on these gels was 30. 11% SDS-PAGE gels were prepared as follows

Reagents (ml)	**Details**	**Separating gel volume (ml)**	**Stacking gel volume (ml)**
Water	None	22	12.7
Acrylamide	30% acrylamide, 0.8% bis	22	2
Lower Tris buffer	1.5M Tris-HCl, pH 8.8	15	0
Upper Tris buffer	0.5M Tris-HCl, pH 6.8	0	5
Ammonium persulfate	20mg/ml in dH_20	0.9	0.3
TEMED	-	0.02	0.02

Table 8: Reagents required in the making of SDS gel electrophoresis (large).

Unlike small gels, the separating and the stacking gels were degassed for 20 minutes before the addition of APS and TEMED. This was done to get rid of the oxygen which inhibits polymerisation. As with the small gels, the samples were mixed with the same 2X loading buffer and incubated at 100°C for 5 minutes prior to loading. The gels were run at 200V in BioRad electrophoresis tanks filled with running buffer.

2.3.4 Coomassie stain and destain

In order to view the protein on gels, they were stained with Coomassie blue containing 20% (v/v) methanol, 5% (v/v) glycerol, 0.25% (w/v) Coomassie Brilliant Blue R250 for 20-60 minutes before being destained with several volumes of destain (10% acetic acid, 30% methanol). The gel was then dried and fixed using the gel drying kit (Promega) and following the manufacturer's instructions. This procedure of staining, destining and drying was not carried out if there was intention of immunoblotting. Instead the protein on the gel was transferred on to a suitable membrane.

2.3.5 Gel analysis using Quantiscan

After Coomassie staining and destaining the gel was dried, analysed and scanned using Quantiscan software (Biosoft, Cambridge). A rectangular box was drawn around every band to measure the density of that band. This box was then moved to the neighbouring uninduced lane that contained the same amount of uninduced protein fraction. Uninduced lane contained total protein from *E. coli* not induced with IPTG. Any band density recorded in this lane was due to leaky expression of the protein and hence was set as background. The background density for every amount of induced protein loaded was subtracted from its respective uninduced lane to get a net band density value.

2.3.6 Transfer conditions

Polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, IPFL 00010) was cut to the size of the gel and then prepared by incubating in methanol until translucent (hydrophilic state), washed in dH_2O for 2 minutes followed by incubation in transfer buffer (25mM Tris, 191mM Glycine, 20% methanol) for a

minimum of 5 minutes. The transfer apparatus was assembled in transfer buffer following the manufacturer's instructions (BioRad). The small gels were transferred at 100V for 1-2 hours at 4°C whereas the big gels were transferred at 40V overnight at 4°C.

2.3.7 Immunoblotting

After completion of transfer, the PVDF membrane was blocked in 1% (w/v) BSA or 5% (w/v) dried skimmed milk prepared in TBS buffer (0.1M Tris pH 7.9, 0.3M NaCl) containing 0.1% (w/v) Triton-X100 or Tween20 (T). This was done to prevent any nonspecific binding of antibodies to the membrane. The membrane was then incubated with the primary antibody prepared in the blocking buffer solution for 1-2 hours at room temperature. For small gels the membrane was washed 3 times for 15 minutes each in TBS-T. For large gels the membrane was washed 5 times for 15 minutes each. Secondary antibody was prepared in the same blocking buffer solution and the membrane incubated with it at room temperature for 1-2 hours. Again the small membranes were washed 3 times for 15 minutes each and the large membranes were washed 5 times for 15 minutes each prior to chemiluminescent staining and development.

2.3.8 Chemiluminescent staining and development of blots

After secondary antibody incubation and subsequent washes, the membrane was incubated in enhanced chemiluminescent (ECL) reagent for a minute. ECL reagent was prepared by mixing solution 1 and 2 together

Solution 1

- 88µl Coumaric acid (0.149g Coumaric acid in 10 ml DMSO) and 200µl luminol (0.443g 3-aminophthalyhydrazine in 10ml DMSO) to 10ml in dH_2O

Solution 2

- 1ml 1M Tris-HCl, pH8.8 and 6.4µl H_2O_2 to 10 ml dH_2O

The PVDF membrane was placed in between an acetate sheet and a laminated piece of card with glow in the dark stickers. The membrane was then exposed to film (Fujifilm X-ray medical film, Super RX) in a closed cassette for as little as 10 seconds to anywhere up to 10 minutes. The film was then developed for a minute in Kodak GBX developer and replenisher (made 1:5 with water) for 1 minute, washed with water and then fixed using Kodak fixer (made 1.25:5 with water) until clear. The film was then allowed to dry before being lined up with the help of the glow in dark stickers and molecular weight markers to tell the exact position of the bands of interest.

2.3.9 Affinity purification of anti-human SULT1E1 IgG in sheep serum on CnBr sepharose coupled to bovine serum proteins.

2.3.9.1 Isolation of human SULT1E1 IgG from sheep serum

Serum from sheep immunized with human SULT1E1 (bleed 2) was diluted in a 1:1 ratio with phosphate-buffered saline solution, PBS buffer (0.1M potassium phosphate, 0.9%NaCl, pH 7.4). An equal volume of saturated ammonium sulfate was added drop wise while mixing on ice. The solution was stirred for a further 30 minutes at room temperature and then centrifuged at 20,000g for 15 minutes at 4°C. Ammonium sulfate suppresses the charge on proteins making them more insoluble . The pellet obtained was resuspended in 10ml PBS buffer and dialysed

overnight in SnakeSkin^{R} pleated dialysis tubing with 3 changes of PBS. 1ml of dialysed IgG was used for further purification on CnBr sepharose column coupled to bovine serum proteins. The rest of IgG samples obtained were aliquoted and stored at -80°C for further use.

2.3.9.2 Preparation of CnBr sepharose coupled to bovine serum proteins

1g of CnBr-Sepharose 4B (Pharmacia) was added to 30 ml of 1mM HCl and washed on a scintered glass funnel over vacuum with 800 ml 1mM HCl. 5mg/ml adult bovine serum and new-born bovine serum (Sigma) were mixed together was made up in a total volume of 10 ml with coupling buffer (0.1M $NaHCO_3$, pH 8.3 containing 0.5M NaCl). Washed Sepharose was added to the bovine serum mixture and left to mix for 2 hours at room temperature or 4°C overnight. The resultant gel was washed on a scintered glass funnel over vacuum with 100 ml coupling buffer. The gel was then transferred to 100 ml of blocking buffer (1M ethanolamine/HCl, pH 8) and mixed for 2 hours at room temperature. Alternate washes in coupling buffer and acetate buffer were carried out 5 times for a total volume of 500ml each. The gel was finally washed with 200ml PBS before packing it on to a column (syringe plugged with glass wool).

2.3.9.3 Affinity purification of anti-human SULT1E1 IgG

The column containing CnBr sepharose coupled to bovine serum protein was initially washed with 5 volumes of PBS. The column was loaded with 1ml IgG preparation and left to stand at room temperature for 1 hour. IgG was eluted with PBS buffer. 1ml fractions were collected and a Lowry assay was performed on them. Fractions containing activity were pooled and the total protein content estimated with Lowry again.

Bovine SULT (bSULT)	**1A1**	**1B1**	**1E1**	**2A1**	**MBP-1A1**	**MBP-2A1**
Primary antibody used	anti-human SULT1A3 (whole protein)	MAP* for MLSPKDILRKDLKLVHG	anti-human SULT1E1 (whole protein)	anti-human SULT 2A1 (whole protein)	Commercial anti MBP¥ (whole protein, Sigma)	Commercial anti MBP (whole protein, Sigma)
	anti minipig SULT1A1 (whole protein)	None	None	None	anti-human SULT1A3 (whole protein)	anti-human SULT 2A1 (whole protein)
Purification	IgG	IgG and CPG	IgG	IgG	serum	serum
Host	Sheep	sheep	Sheep	sheep	rabbit	rabbit
Dilution	1:5000 (both antibodies)	1:1000	1:10,000	1:10,000	1:10,000	1:10,000
Blocking agent	1% BSA	1% BSA	1%BSA	5% Milk	5%milk	5% milk
Wash buffer	TBS-Triton X	TBS-Triton X	TBS-Triton X	TBS-Triton X	TBS-Tween 20	TBS-Tween 20
Secondary antibody	rabbit anti goat HRP	rabbit anti goat HRP	rabbit anti goat HRP	rabbit anti goat HRP	goat anti rabbit HRP	goat anti rabbit HRP
Dilution	1:5000	1:5000	1:5000	1:5000	1:20,000	1:20,000
References for primary antibodies	(Richard et al., 2001)	(Riches et al., 2009)	(Rubin et al., 1999)	(Forbes et al., 1995)	(Richard et al., 2001)	(Forbes et al., 1995)

Table 9: Blotting conditions for the detection of bovine SULT isoforms.

*Anti-human SULT antibodies were used in the detection of bSULT isoforms. *MAP stands for multiple antigenic peptides. ¥MBP stands for maltose binding protein. Anti MBP was used along with anti-human SULT1A3 and anti-human SULT2A1 in the detection of MBP tagged proteins. All the secondary antibodies used are horseradish peroxidise conjugates (HRP) that were purchased from Sigma-Aldrich.*

2.4 Cloning and expression of recombinant bovine SULT isoforms in a microbial expression system

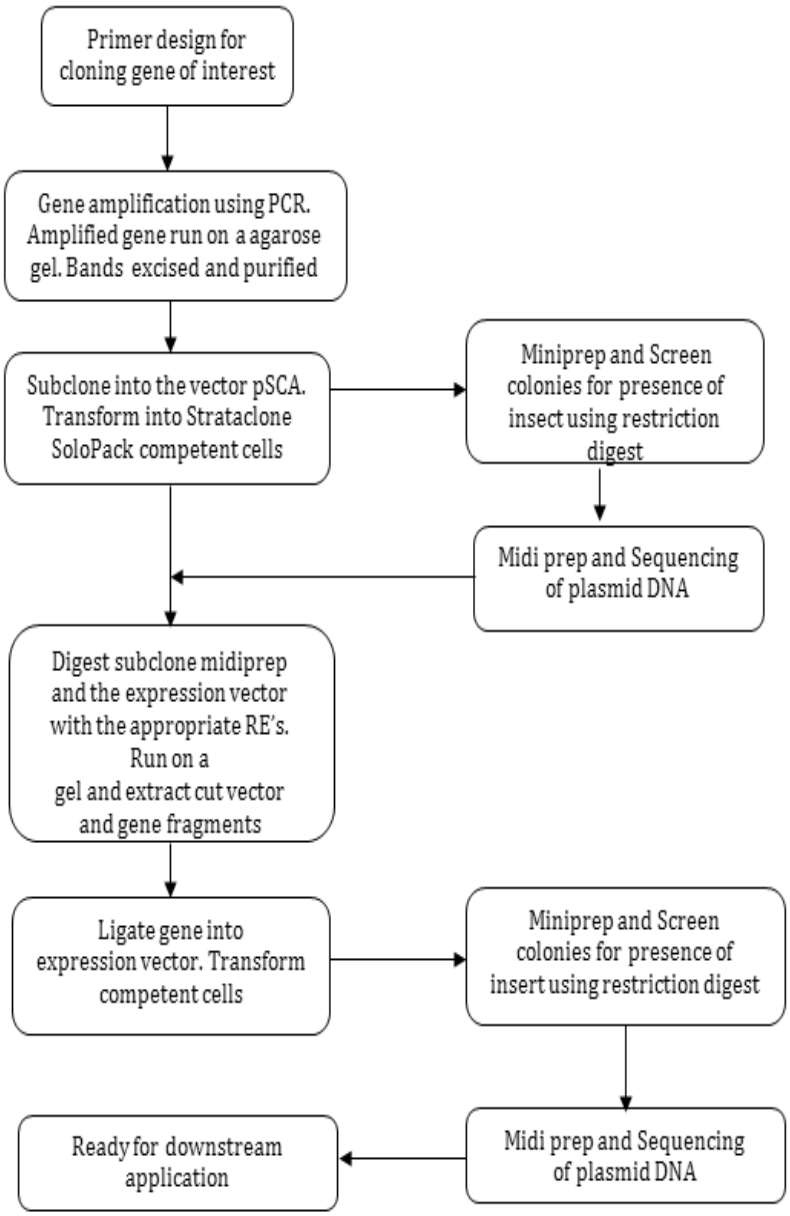

Figure 10: Overview of the cloning procedure

2.4.1 Image Clones

Image clones containing 5 different bovine sulfotransferase genes were purchased from Source BioScience plc, Nottingham. The table below lists information on the NCBI accession numbers, vectors used, the strain, sex, age and tissue of the cattle whose mRNA was used in making of the Image clone.

Image Clone ID	Accession #	Bovine isoform	Vector	Strain	Sex, Age and Tissue information
7945971	BC102274	SULT1A1	pCMV.SPORT6.0	Cross bred x angus	Female, 6months old, Ileum
7989385	BC102939	SULT1E1	pCMV.SPORT6.0	Hereford	Male, 6 month old, Liver
7944770	BC102208	SULT1B1	pCMV.SPORT6.0	Cross bred x angus	Female, 6months old, Ileum
8013931	BC112644	SULT2A1	pCMV.SPORT6.0	Hereford	Male. 7 years old, Testis
8478800	BC126756	SULT2B1	PExpress1	L1 Hereford	Female, 6months old, skin

Table 10: Information on the Image clone DNA used in the cloning of bovine SULTs

2.4.2 Vectors and Tags

Untagged SULTs were cloned into the expression vector pET-17b and expressed in BL21 (DE3) pLysS. Along with the untagged expression, bSULT1A1 and bSULT2A1 were also expressed as a maltose binding protein (MBP) fusion. In the pMAL-c2x vector used for the expression of the MBP fusion protein, the cloned gene is

inserted downstream from the *MalE* gene of *E. coli* that encodes MBP resulting in the expression of an MBP fusion protein. The TB1 strain of *E. coli* was used for expression of MBP fusion proteins.

2.4.3 Primer design

Some basic rules were applied to primer design . Primers were designed such that they had a similar T_m, ideally between 60°-70°C to allow binding to the template. In addition to this, primers were designed such that they did not form any dimers or secondary structures. They were also initiated with either a G or a C in order to enhance ligation efficiency into subsequent subcloning vectors.

Firstly, the Expasy translation tool (http://expasy.org/tools/dna.html) was used to choose the appropriate reading frame and hence determine the correct coding region of the gene of interest. Once the coding region was selected, restriction enzymes were chosen. Restriction enzymes (REs) that produced sticky ends were preferred given their ease to work with. Using the web based tool, webcutter (http://users.unimi.it/~camelot/tools/cut2.html) REs that did not cut the insert but cut the expression vector at unique sites were chosen. These sites were then incorporated into the primer sequence.

Primers sequences with similar T_ms were designed with the aid of Invitrogen Oligoperfect http://tools.invitrogen.com/content.cfm?pageid=9716. Desalted primers were purchased from Sigma Genosys.

2.4.4 Amplification of cDNAs encoding bovine SULTs

As the first step towards cloning, cDNAs encoding different SULT isoforms (Table 1 except bSULT2B1) were amplified from the Image clone using the primers

designed. After optimization of annealing temperatures, a 50 µl PCR reaction was set up. Given below is a typical PCR reaction.

Reactants	Stock concentration	Final assay concentration	Supplier	Volume (µl)
Buffer	10X	1X	Bioline	5
MgCl2	50mM	2mM	Bioline	2
dNTP mix	10mM each dNTP, 40mM total	0.02mM	Promega	1
Forward primer	100µM	0.2µM	Sigma Genosys	1
Reverse primer	100µM	0.2µM	Sigma Genosys	1
Template DNA	variable	30-40ng	NA	variable
BIO-X-ACT polymerase	4U/µl	4u	Bioline	1
Nuclease free water	NA	NA	Ambion	variable

Table 11: PCR reaction mixture for amplification of bovine Sulfotransferases.

NA stands for not applicable

Temperature/time	# Cycles
95°C, 5min	x1
95°C, 30sec X°C, 30 sec 68°C, 1min	x35
68°C, 7min	x1
6°C, HOLD	-

Table 12: Thermocycler program settings used for PCR

PCR products were subsequently run on a 0.1% agarose gel, excised and purified using the QIAquick gel extraction and purification kit (Qiagen)

Gene	Nt	primer name	Vector	Tag	Sigma Tm (°C)	Actual Tm used (°C)	Restriction enzymes	Sequence 5'-3'
bSULT1A1	29	pET-17b bSULT1A1for	pET-17b	None	74.1	79.3	*NdeI*	CATATGGAACTGATCCAGGATACCTCCCG
bSULT1A1	25	pET-17b bSULT1A1rev	pET-17b	None	74.5		*HindIII*	AAGCTTTCACAGCTCCCAGCGGAAG
bSULT1A1	25	MBP-bSULT1A1for	pMAL-c2x	MBP	67.8	67.8	*XmnI*	GAAGGATTTCAATGGAACTGATCCA
bSULT1A1	24	MBP-bSULT1A1rev	pMAL-c2x	MBP	74.3		*HindIII*	AAGCTTTCACAGCTCCCAGCGGAAG
bSULT2A1	28	pET-17b bSULT2A1for	pET-17b	None	67.9	65.4	*NdeI*	CATATGACAGGAAAGTTTCTGTGGTTTG
bSULT2A1	24	pET-17b bSULT2A1rev	pET-17b	None	69.6		*HindIII*	AAGCTTTTATTCCCACGCGAACAG
bSULT2A1	33	MBP-bSULT2A1for	pMAL-c2x	MBP	73.2	67.1	*XmnI*	GAAGGATTTCAATGACAGGAAAGTTTCTGTGGT
bSULT2A1	24	MBP-bSULT2A1rev	pMAL-c2x	MBP	69.6		*HindIII*	AAGCTTTTATTCCCACGCGAACAG
bSULT2A1	29	Yeast_bSULT2A1for	pYES2	None	68.2	63.2	*HindIII*	AAGCTTATGACAGGAAAGTTTCTGTGGTT
bSULT2A1	24	Yeast_bSULT2A1rev	pYES2	None	68.2		*EcoRI*	GAATTCTTATTCCCACGCGAACAG
bSULT1B1	29	pET-17b bSULT1B1for	pET-17b	None	68.2	65.7	*NdeI*	CATATGACTTCTCCAAAAGATGTCCTGAG
bSULT1B1	29	pET-17b bSULT1B1rev	pET-17b	None	69		*HindIII*	AAGCTTTCAAATCTCTGTACGGAACTGAA
bSULT1E1	29	pET-17b bSULT1E1for	pET-17b	None	68.6	68.6	*NdeI*	CATATGAGTTCTTCCAAACCATCCTTTTC
bSULT1E1	31	pET-17b bSULT1E1rev	pET-17b	None	68.8		*EcoRI*	GAATTCCTAGATCTTAGTTCGGAACTTCAGG

Table 13: Primers used in the cloning of various tagged and untagged isoforms of bovine sulfotransferases.

(All primers purchased from Sigma Genosys)

2.4.5 Sub cloning (pSCA-Amp/Kan, Stratagene)

2.4.5.1 Poly A tail addition

The Strata clone PCR cloning vector mix used contains vector pSCA-Amp/Kan that has two DNA arms which have a modified uridine (U) overhang. Taq amplified products that have a 3' adenosine overhang can be efficiently ligated into these vectors. Hence a Poly 'A' tail was added to the purified PCR products before ligation. The reactants were assembled as follows. The reaction was carried out at 72°C for 30 min

Reactants	Stock concentration	Final assay concentration	Supplier	Vol (µl)
Colourless GoTaq buffer	5X	1X	Promega	4
$MgCl_2$	25mM	2.5mM	Promega	2
dATP	2mM	0.2mM	Promega	2
Go taq polymerase	5u/µl	5u	Promega	1
Purified PCR product	-	-	-	11
Total	**-**	**-**	**-**	**20**

Table 14: Details of reactants used in poly A tail addition

2.4.5.2 Ligation and Transformation

Following Poly 'A' tail addition, the PCR product was ligated into pSCA using the StrataClone protocol for ligation (StrataClone PCR Cloning Kit, Stratagene, 240205). The construct was subsequently transformed into StrataClone SoloPack competent cells using the StrataClone protocol for transformation. Colonies were selected from the transformed plates the next day and used to inoculate a 5ml

small culture. In order to obtain DNA from these colonies for subsequent screening, minipreps were done according to the manufacturer's instructions (Qiagen miniprep kit).

2.4.5.3 Restriction endonuclease digest to screen colonies for the presence of insert

A digest using miniprep DNA as template and EcoR1 restriction endonuclease was performed to screen colonies for positive clones. EcoR1 was chosen for the digest because it contained restriction sites in the multiple cloning region and it flanked either side of the PCR product insertion site in pSCA. A single product around 1000 base pairs was expected to be seen in positive clones. A typical digest is shown below. Once the positive clones were identified, 500 µl from the positive small culture was used to inoculate a 50 ml culture. This 50 ml culture was used for subsequent midiprep to generate large quantities of plasmid DNA from the positive clones for sequencing. A typical digest is shown below

Reactants	**Volume (µl)**
10X BSA	1
10 X buffer	1
DNA	2
ECoRI	0.5
Nuclease-free water	5.5

Table 15: Typical Restriction endonuclease digest.

It was carried out to screen for positive colonies containing the desired pSC-A insert

2.4.5.4 Sequencing

Sequencing of insert was carried out to ensure that the DNA is free of any nucleotide base pair changes that could affect gene expression. The pSCA construct midiprep was sent to be sequenced. Sequencing was performed by (The Sequencing Service, University of Dundee, Scotland , www.dnaseq.co.uk) using Applied Biosystems Big dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Some of the sequencing was also performed by the DNA Analysis Facility Ninewells Hospital and Medical School, Dundee. pSC-A constructs were sequenced using T3 and T7 primers, pET-17b constructs using T7 and T7 term primers and pMAL-c2x constructs sequenced using *MalE* primers.

Primer name	**Sequence (5' – 3')**	**Used for**
MalE Forward	GGTCGTCAGACTGTCGATGAAGCC	pMAL-c2x_bSULT2A1 pMAL-c2x_bSULT1A1
MalE reverse	CGCCAGGGTTTTCCCAGTCACGAC	pMAL-c2x_bSULT2A1 pMAL-c2x_bSULT1A1
T7	TAATACGACTCACTATAGGG	All pET-17b, pSC-A constructs
T3	AATTAACCCTCACTAAAGGG	pSC-A constructs
T7 terminator	CTAGTTATTGCTCAGCGGT	pET_17b constructs

Table 16: Forward and reverse primers used for sequencing inserts of given constructs.

2.4.6 Cloning and expression

2.4.6.1 Restriction digests and dephosphorylation of the expression vector.

Following satisfactory results from analysis of the sequencing data, preparations were made to insert the gene of interest into the expression vector used. Firstly, the midi prep subclone and the expression vector were cut with the appropriate restriction enzymes (refer to the section on primer design). The products were run on a 0.8% agarose gel. Ideally 3 distinct bands one corresponding to the cut expression vector and other to the insert and cut pSCA were seen. Bands corresponding to the gene of interest and the expression vector was viewed and cut under UV light. DNA from cut bands was extracted and purified using QIAquick PCR purification kit.

2.4.6.2 Dephosphorylation, ligation and transformation of the expression construct into competent E .coli

Cut expression vector was dephosphorylated using the enzyme CIAP (**C**alf **I**ntestine **A**lkaline **P**hosphatase). This was done to prevent religation of the expression vector without the insert. The dephosphorylation was set up as below and the reaction was allowed to proceed at 37°C for an hour. This was followed by Qiagen PCR clean up.

Reagents	Volume (µl)
DNA (Elution from gel clean up)	30
CIAP	2
CIAP buffer 10X	4
Nuclease-free water	4
Total	**40**

Table 17: A typical dephosphorylation reaction set up

Typically, several ligation reactions containing variable amount of insert were carried out to increase the chances of successful ligation. All the ligation reactions were carried out at 4°C overnight as these were established to be optimum conditions in previous experiments. The ligated products were then transformed into competent *E. coli* using standard protocol described in section 2.4.7.1

	Volume (µl)				
Dephosphorylated vector	1	1	1	1	1
Insert	0	2	4	6	8
T4 ligase buffer (10X)	2	2	2	2	2
T4 Ligase	1	1	1	1	1
Nuclease -free water	16	14	12	10	8
Total	**20**	**20**	**20**	**20**	**20**

Table 18: A typical ligation reaction set up

A ligation reaction set up with increasing amount of the insert to increase the efficiency and ensure successful ligation.

Upon transformation, all the procedure such as midiprep of DNA from positive clones and subsequent sequencing of the expression construct was carried out in the same way as it was for pSCA (subclone) constructs with the only exception being the restriction enzyme digest. Where possible a double digest was performed, i.e. when two restriction endonucleases from the same manufacturer had 100% compatibility in the same buffer, they were used together in one digest. When this was not the case, sequential digests were performed. Here one digest was performed with one endonuclease and the resulting product was cleaned up using a PCR clean up column (Qiagen) and used as a template for the reaction with the second endonuclease.

Glycerol stocks were made for future use from all the expression constructs created as described in section 2.4.7.3.

2.4.6.3 Expression of recombinant bovine SULT isoforms

Glycerol stocks of recombinant bovine SULTs were streaked onto plates containing 100µg/ml ampicillin. Colonies were formed on these plates after an overnight incubation at 37°C. A randomly chosen colony was used to inoculate a 5 ml LB broth containing 100µg/ml ampicillin at 37°C overnight. 2 ml of this small culture was used to inoculate a 200 ml culture containing 100µg/ml ampicillin. The culture was grown at 37°C in a shaking incubator until it reached an optical density (OD) of 0.6-0.9. At this point it was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and shifted to 30°C overnight. The protein was harvested by using two different methods. One used lysozyme and the other sonication for cell lysis.

Isoform	IPTG (mM)	$OD_{induction}$
bSULT1A1	1	0.9
bSULT1B1	0.5	0.6
bSULT1E1	1	0.6
MBP_bSULT2A1	1	0.65

Table 19: Conditions used for the expression of various bovine SULTs

2.4.6.4 Making cell free extract (lysozyme)

Induced bacterial cultures were spun at 900g in 50 ml centrifuge tubes. The pellet obtained was resuspended in 2ml of 40mM Tris/2mM EDTA, pH 8. 100µl of 10mg/ml freshly made lysozyme was added to each tube and incubated for 15 minutes at room temperature. Further, 100µl of 5% BRIJ 58/200mM $MgSO_4$ was added to the tube followed by 15 minute incubation at room temperature. In order to get rid of any genomic DNA, 10µl of RNase free DNase was added and the mixture was incubated at 37°C for 3min and then at room temperature for 2min. The mixture was then divided into 1.5ml eppendorf tubes and spun at 6500g for 5min. A Lowry assay was carried out on the supernatant to measure the amount of protein obtained.

2.4.6.5 Making crude extract (sonication)

Induced bacterial cultures were spun at 4000g for 20 minutes. The pellet obtained was resuspended in 2ml column buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA). The samples were placed on ice and sonicated in 4 short pulses of 15 seconds (4X15) each pulse followed by a minute incubation on ice to prevent protein degradation caused due to excess heat. The number of sonication pulses

and the duration of the pulse were optimised for every isoform. 4x15 second pulses were found to be optimum for all isoforms used. Prior to sonication, protease inhibitors (Protease inhibitor cocktail tablets, Roche) were added to the lysate. 100µl of 25X stock solution made by dissolving 1 tablet in 2ml water, was added to the lysate to prevent protein degradation resulting due to protease activity. The sonicated mixture was spun at 9000g for 30 min to pellet the cell debris. A Lowry assay was performed on supernatant obtained.

2.4.7 Standard techniques used in cloning

2.4.7.1 Transformation

50-100µl of competent cells were incubated with 50-500ng plasmid DNA and left on ice for 20min. The mixture was then heat shocked at 42°C for 45seconds and then placed on ice again for 2min. LB broth containing no antibiotic was added to the mixture and shaken in a 37°C incubator for at least an hour. After heat shock, SoloPack competent cells from Strataclone were resuspended in 250µl LB as per the manufacturer's instructions whereas competent BL21 (DE3) pLysS, JM109 and TB1 were resuspended in 1ml LB. The cells were centrifuged at 10,000g for 1min. 5 and 100µl of competent cells were plated on LB plates containing 100ug/ml ampicillin. These plates were incubated overnight at 37°C.

2.4.7.2 Making competent cells

Supercompetent JM109, BL21 (DE3) pLysS, and TB1 cells were made using standard protocols. A 10ml overnight culture of non-competent cells was used to inoculate a 200ml culture of LB broth. It was incubated at 37°C until an OD of 0.3-0.5 was reached. The inoculant was chilled on ice and then spun at 3000 rpm for 5min at 4°C. The pellet was resuspended in 80 ml of TFBI buffer and kept on ice

for a further 20 min. Cells were once again pelleted and resuspended in 8ml TFBII. They were kept on ice for an hour before aliquoting them into pre chilled microfuge tubes. Resultant competent cells were snap frozen in liquid nitrogen and stored at -80°C.

TFBI	
Potassium acetate	30mM
Rubidium chloride $RbCl_2$	100mM
Calcium chloride $CaCl_2.2H_2O$	10mM
Manganese chloride $MnCl_2.4H_2O$	50mM
Glycerol	15ml
dH2O	80ml
Adjust to pH5.8 with 0.2M acetic acid and filter sterilise	

TFB II	
MOPS	10mM
Rubidium chloride $RbCl_2$	10mM
Calcium chloride $CaCl_2.2H_2O$	75mM
Glycerol	15ml
dH_2O	80ml
Adjust to pH 6.5 with 1M KOH and filter sterilise	

Table 20: Content of buffers used in the making of competent cells

2.4.7.3 Making glycerol stocks

A single positive clone containing the desired construct was used to inoculate 5ml LB broth (with antibiotic). The culture was grown at 37°C overnight and aliquoted into cryovials. 80% (v/v) glycerol was added to the culture in a ratio of 1:4. The cryovials were then snap frozen in liquid nitrogen and stored at -80°C until further use. A small amount of the glycerol stock was scraped from the top of the tube (without thawing) using an inoculation needle with a loop as and when needed.

2.4.8 Primary and secondary structure analysis of bovine SULTs

In order to compare the primary structure of bovine SULTs with that of human (and mouse Sult1e1), amino acid sequences were obtained from NCBI. (http://www.ncbi.nlm.nih.gov/nuccore). The sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). Critical residues were subsequently identified. Crystal structure files of proteins were downloaded from protein data bank (http://www.pdb.org/pdb/home/home.do). The structure file was downloaded in pdb format and opened using a structural tool called Discovery Studio Visualizer (DS Visualizer 3.1, Accelrys software, Cambridge). This software was also used for analysis of the downloaded structures.

2.5 Protein Purification: A two-step procedure for purification of recombinant bSULTs expressed in *E. Coli*

2.5.1 General

Purification of SULTs from crude extracts was carried out using ion exchange and affinity chromatography with Q sepharose and ADP agarose columns respectively. Ammonium sulfate precipitation, a crude form of purification is usually the first step in any purification protocol. ADP agarose columns are the only ones used here that bind SULTs specifically. Buffer A (50mM Tris, pH 8, 78µl mercaptoethanol/L) was the primary buffer used in this purification. Buffer B containing 1M NaCl in buffer A was also used.

2.5.2 Ammonium sulfate precipitation

Cell free extract obtained by expressing recombinant bovine sulfotransferases in *E. Coli* were initially subjected to ammonium sulfate precipitation also termed as the 'salting out' reaction. Proteins precipitate according to their solubility at different salt concentrations. A two stage ammonium sulfate precipitation was carried out. Finely ground solid ammonium sulfate was added to the cell free extract at 35% saturation (0.197g/ml) at 4°C over a period of 40 min. The mixture was then left to incubate on ice for 1 hour. It was then centrifuged at 16000g for 15 minutes. The pellet was discarded and more ammonium sulfate at 70 % final saturation (0.245g/ml) was added to the supernatant. The procedure was repeated exactly as stage 1. After centrifugation, the supernatant was discarded and the pellet containing the precipitated protein was resuspended in 5ml buffer A The solution was dialysed overnight in SnakeSkin^{R} pleated dialysis tubing with three changes of buffer A to remove salt. A Lowry assay was carried out to measure the amount of protein present in the mixture after ammonium sulfate precipitation

2.5.3 Q Trap ion exchange chromatography

Ion exchange chromatography was used in the second stage of protein purification. The separation of proteins is based on the reversible binding of their charged molecules to oppositely charged groups attached to an insoluble matrix. This stage of purification was carried out using a strong anion exchange column. An anion exchange column contains positively charged groups and binds negatively charged molecules in the protein mixture. A 5ml Hi-Trap Q sepharose (GE healthcare) column attached to FPLC AKTA purification system was used. Prior to loading of protein, the column was washed with 30 ml high salt buffer B to

get rid of any bound protein. The column was then re equilibrated with 60 ml Buffer A. Protein up to 50mg/ml was injected manually on to the column via an injection port on the AKTA. Buffers A and B were mixed appropriately in a gradient mixer on the AKTA to generate a salt gradient of 0-1M NaCl that was passed onto the column over 150ml. A fraction collector was used to collect protein eluted in 3ml fractions. All the fractions were run on a 15% SDS PAGE gel and assayed for SULT activity. Fractions containing the protein of interest were pooled together and concentrated to 3ml using an Amicon concentrator (Amicon) and following the manufacturer's instructions. The sample was dialysed overnight with 3 changes of buffer A. A small fraction of sample was withdrawn at every stage of purification and used for the determination of protein enrichment and purification.

2.5.4 ADP agarose affinity chromatography

2.5.4.1 Pouring the column

15mg of 3'5'-ADP agarose (Sigma) were resuspended in buffer A and poured into the column. Agarose beads were washed with excess buffer that was allowed to drain from the column. The column was left to stand for an hour in order to settle the agarose. A stopper from the top of the column was pressed down gently to avoid forming any air bubbles. The newly poured column was equilibrated with buffer A. At the end of every run, the column was always washed with 2M NaCl to get rid of any bound protein.

2.5.4.2 Running the column

Before loading the protein, the column was washed with 90ml buffer B to get rid of bound protein. It was then re equilibrated with 130ml buffer A. Protein was

injected manually onto the column via the injection port on the AKTA. Once onto the column, the protein was washed at a flow rate of 2ml/min with buffer A containing 0.05M KCl for a period of 15 minutes. This was done to get rid of any nonspecific binding to the column. 100μM PAPS in 5ml buffer A was used to elute the protein. SULTs bind ADP agarose with a lower affinity compared to PAPS. Hence when PAPS is added to the ADP agarose column the SULTs bound to the column dissociate from it and bind PAPS. The column was then washed with 0.05M NaCl for 15 minutes followed by 0.5M NaCl for another 15 minutes in order to elute the SULT of interest. Once again fractions were analysed on a 15% SDS PAGE gel and assayed for SULT activity. Fractions containing the desired protein were pooled together and concentrated using the Amicon concentrator. The concentrated sample was then desalted on a PD-10 column (GE healthcare) according to the manufacturer's instructions. The purified protein was aliquoted and snap frozen in liquid nitrogen. A Lowry assay was performed to measure the amount of purified protein. The purified protein was then run on a 15% SDS PAGE gel alongside pooled crude extract, ammonium sulfate and Q trap fractions to assess its purity. Activity assays were carried out on fractions pooled after each stage in order to determine fold purification.

2.6 In vitro metabolism of 17α/β-estradiol in microsomes, cytosol and hepatocyte samples

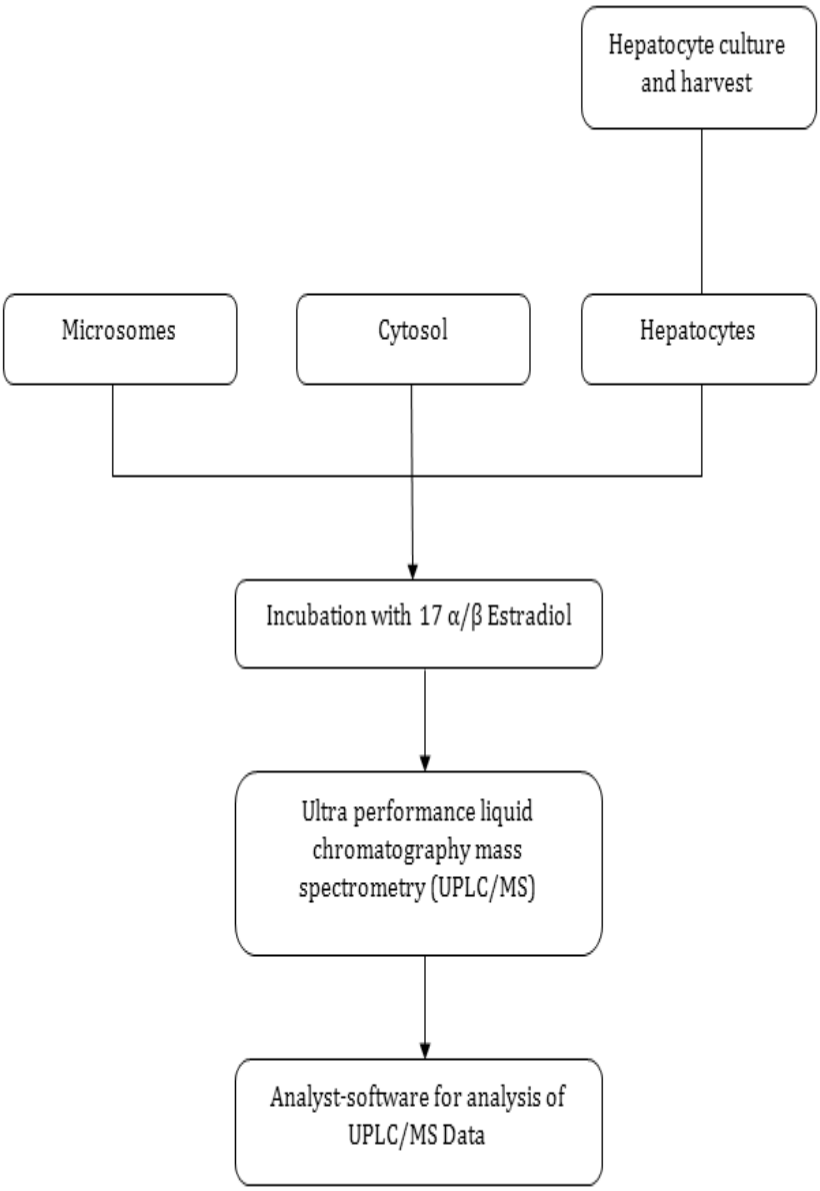

Figure 11: Conjugative metabolism of 17β-estradiol.

Outline of the procedure employed to study conjugative metabolities of 17β-estradiol in microsomes, cytosol and hepatocytes. All the reactions were carried out in 96 well plates and set up in triplicates

2.6.1 Liver procurement and hepatocyte preparation:

A segment of liver tissue was obtained from a 14-month old heifer of an Angus Cross (mixed) breed (Pfizer Inc.), perfused to remove blood and placed on ice prior to hepatocyte isolation. Cryopreserved hepatocytes were prepared from this tissue by Piedmont Research Centre (Lot No. Bov-10-0001, Charles River Discovery & Imaging Services Morrisville, NC) and stored under liquid nitrogen.

2.6.2 Hepatocyte harvest

Hepatocytes were thawed and cell suspensions transferred to pre-warmed (37°C) Clonetics® HMM™ media supplemented with 10^{-7}M insulin and 10^{-7}M dexamethsome (Hepatocyte Maintenance Media (HMM) with UltraGlutamine-1, Lonza Lot No. 0000176054 Catalog No. CC-3197) containing 30% Percoll (Sigma Chemical Company, St. Louis, MO) and centrifuged at 130 x g for 20 minutes at room temperature. The supernatant containing non-viable cells was carefully aspirated, and the resulting pellet was washed with pre-warmed HMM, diluted to approx. 2 × 10^6 viable cells/mL and cell viability was assessed using trypan blue dye exculsion methods.

2.6.3 Incubation of hepatocytes with 17α/β-estradiol

Stock 17β-estradiol was prepared at a 2 times final concentration in pre-warmed HMM buffer. To start the reactions, a 50 µL aliquot of the hepatocyte suspension was added to a 50 µL aliquot of substrate solution into a 1 mL, 96-well round-bottom plate (Costar) resulting in a cell concentration of 1x10^6 cells/mL and incubated in a 37 °C incubator water bath for 3 minutes. The reaction was terminated with the addition of 100µL of ice cold 80:20 acetonitrile: methanol

containing internal standard (10ml of 5µg/ml 7-hydroxycoumarin-glucuronide was added to 75 ml acetonitrile: methanol and 10ml 200µg/ml 7-hydroxycoumarinsulfate was added to 10 ml acetonitrile: methanol) to 100µL of cell suspension. The mixture was centrifuged at approximately 3000g for 15 minutes at 4°C, 80µL of the supernatant was transferred to a clean 96-well v-bottom 0.5mL plate (Costar) and evaporated to dryness under nitrogen gas using a TurboVap® concentration workstation (Caliber Life Sciences). The samples were resuspended in 100µL of 95:5 water: acetonitrile containing 0.1% formic acid and analysed by LC-MS multiple reaction monitoring for estradiol glucuronide or sulfate.

2.6.4 Incubation of microsomes and cytosol with 17α/β-estradiol

2.6.4.1 Microsome incubations

Reactants (-drug) were incubated with alamethicin on ice for approx. 15 min. Alamethicin introduces pores in the membrane, thus facilitating the passage of drug through the microsomal membrane to reach the UGT active site. After incubation, the plate transferred to a 37°C water bath and the drug (17α/β-estradiol) was added. When the temperature in wells in the plate reached 37°C, reaction was started by the addition of UDPGA (uridine 5'-diphospho-glucuronic acid). Control reactions containing no UDPGA were also set up in triplicate. The reaction was terminated by adding 100µL of 25% formic acid in water. 20µl of 5µg/ml 7-hydroxycoumarin-glucuronide were added as internal standard (IS) to every reaction. The samples were centrifuged at 3000g for 15 minutes before being injected on the UPLC/MS. The reactants for the incubation were set up as follows:

Ingredients	Final in assay
Bovine Liver Microsomes	Variable
UDPGA	0.5mM
Alamethicin	50ug/mg protein
$MgCl_2$	1mM
Drug	Variable
Incubation volume	**200µl**

Table 21: Example of a reaction set up for bovine liver microsome incubation with 17α/β-estradiol

2.6.4.2 Cytosol incubations

All reactants given above were assembled at 37°C. Reactions were started by the addition of 20µl PAPS and stopped with 100µL acetonitrile containing 20µL of 200µg/ml 7-hydroxycoumarinsulfate as an internal standard. After termination of reaction, 180µl from each well were transferred to a fresh 2ml vial. The vials were then spun at 10,000g in a minicentrifuge for 15 minutes. The supernatant was transferred back to a fresh clean plate and samples were dried in TURBOVAP nitrogen dryer prior to resuspending them in 100µL, 0.1% aqueous formic acid. All this was done to improve the signal intensity upon injection into the UPLC/MS system.

Ingredients	Final in assay
Incubation Volume	200uL
Bovine Liver cytosol	Variable
PAPS	0.1mM
Drug	Variable

Table 22: Example of a reaction set up for bovine liver cytosol incubation with 17α/β -estradiol

Data obtained from UPLC/MS was analysed using Analyst 1.4.2 (AB Sciex) software package.

2.6.5 UPLC/ Mass Spectrometry Conditions for Glucuronidation and sulfation reactions

2.6.5.1 Chromatography

Ultraperformance liquid chromatography was performed on ACQUITY™ UPLC™ SYSTEM (Waters Corp., Milford, MA, USA). ACQUITY UPLC™ HSS T3 column (100 X 2.1mm, 1.8µM) was used for glucuronidation and ACQUITY UPLC™ BEH shield RP18 column (50 x 2.1mm, 1.7µM) was used for sulfation. Chromatographic separations were achieved with a gradient elution using the mobile phase composed of water and acetonitrile (both containing 0.1% formic acid). The chromatographic separation for the ACQUITY UPLC™ HSS T3 column was started at 10% acetonitrile for 0.1min and then ramped to 70% within 5min and then returned to 10%. (table 23) For the ACQUITY UPLC™ BEH shield RP18 column, the chromatographic separation was started at 10% acetonitrile for 2min and then

ramped up to 70% acetonitrile within 0.1 min (table 24). For both the columns, the flow rate was set at 0.6ml/min and the injection volume was 10µl.

Time (min)	Flow rate (ml/min)	%A2	%B2
Initial	0.6	90	10
0.1	0.6	90	10
5	0.6	30	70
5.1	0.6	30	70
5.2	0.6	90	10
10	0.6	90	10
60	0.6	90	10

Table 23: Details of chromatographic conditions used with the UPLC/MS for glucuronidation reactions.

Time (min)	Flow rate (ml/min)	%A2	%B2
Initial	0.6	90	10
0.1	0.6	90	10
2	0.6	90	10
2.1	0.6	30	70
2.2	0.6	30	70
4	0.6	90	10
60	0.6	90	10

Table 24: Details of chromatographic conditions used with the UPLC/MS for sulfation reactions.

2.6.5.2 Mass Spectrometry

Mass spectrometric detection was carried out on a triple quadruple instrument (API 4000, Applied Biosystems SCIEX) with an electrospray ionisation (ESI) interface. The ESI source was set in negative ionization mode and the quantification mode was multiple reaction monitoring (MRM). The following precursors to product ion transitions were used for MRM transition: 7-hydroxycoumaringlucuronide (internal standard), *m/z* 336.9→161; 7-hydroxycoumarinsulfate (internal standard), *m/z* 241→161; 17β-estradiol-3-glucuronide, *m/z* 447.2→271.10; 17β-estradiol-17-glucuronide, *m/z* 447.2→ 271.10; 17β-estradiol-3-sulfate, *m/z* 351→271. All data collected was acquired and processed using the software Analyst® (Applied Biosytems SCIEX)

3 Characterization of recombinant bovine sulfotransferases

3.1 Introduction

The development of new therapeutic drugs requires a thorough preclinical testing stage where the pharmacodynamic, pharmacokinetic and toxicological properties of the candidate are evaluated. This preclinical investigation can involve the use of both *in vitro* as well as *in vivo* approaches. An ideal *in vitro* model should accurately resemble the biotransformation of a particular drug in the tissue of interest. Although other organs may also be involved in the biotransformation of drugs, the liver is the most predominant organ in which the majority of foreign compounds are metabolised. Hence several *in vitro* human liver models such as recombinant enzymes, microsomes, cytosol, S9 fraction, liver cell lines, hepatocytes, liver slices and perfused liver have been developed in the past (Brandon et al., 2003; Ekins et al., 2000). A great advantage of the availability of such *in vitro* model systems is that they allow the study of drug biotransformation in a system that is less complex than that found *in vivo*.

One way of understanding a complex process like drug metabolism is to take the reductionist viewpoint and isolate the smallest possible unit which in this case is the drug metabolising enzyme itself. This is where enzymes generated using recombinant DNA technology finds their benefit. They can be used for isoform specific drug biotransformation studies (Ekins et al., 2000). This information is particularly useful since in combination with expression profile of DMEs in various tissues will allow drug companies to make accurate prediction on the metabolic fate of a particular drug *in vivo.* Companies such as Simcyp already has a technology platform which incorporates data on quantification of drug metabolising enzymes in various tissues to help predict the metabolic fate of a drug *in vivo* (Jamei et al., 2009). Recombinant enzymes can also be purified and subsequently crystallised in the presence of the drug of interest to reveal

important structural features involved in drug binding and catalysis, thus aiding the design of better and safer drugs. Drug-drug interactions (DDIs) can lead to severe side effects, refusal of approval, severe prescribing restrictions and withdrawal of drugs from the market. They usually result from the action of one drug affecting the metabolism uptake or the removal of another drug (Friedberg, 2000). With respect to DDIs, UGTs are the most investigated phase 2 enzymes. Some common DDIs include inhibition of acetaminophen glucuronidation by drugs such as propranolol and ranitidine. Valproic acid inhibits the glucuronidaton of the anticonvulsant drug carbamazepine and the anti-viral drug AZT. Rifampin is known to increase the glucuronidation of codeine whereas estrogen containing oral contraceptives increase the glucuronidation of acetaminophen (Kiang et al., 2005). As compared to glucuronidation, DDIs affecting sulfation are not very well researched due to limited availability of recombinant enzymes and because of lack of substrate/inhibitor specificity. Compounds such as mefanamic acid and quercetin have been identified as potent inhibitors and drugs such as phenobarbital and rifampicin are known to induce the action of sulfotransferases, however no clinical drug-drug interaction resulting from their use has yet been reported (Bjornsson et al., 2003). Availability of individual isoforms of recombinant drug metabolising enzymes will allow DDIs as a result of biotransformation of one or more compounds to be studied at the enzyme level, thus aiding the design of drugs with lesser side effects.

Genetic polymorphisms in drug metabolising enzymes can result in enhanced or decreased glucuronidation activity in patients possessing the allelic variants. *UGT1A1*28* is an allelic variant of *UGT1A1* and is associated with reduced

elimination of SN-38 a toxic metabolite of the anti-cancer drug irinotecan (Iyer et al., 2002). Similarly genetic polymorphism in *SULT1A3* has been implicated in changes of action of levosalbutamol, an R-enantiomer of the β2-adrenergic receptor agonist salbutamol (Boulton and Fawcett, 2001). Mutant recombinant enzymes can be generated to include these allelic variants and potential drugs can be tested on them to assess the activity relative to the wild type. Upon successful extrapolation to *in vivo* conditions in the clinic, it will enable patients to be segregated on basis of their potential response to treatment.

In the following series of experiments, bovine sulfotransferase (SULT) isoforms such as SULT1A1, SULT1E1, SULT1B1 and SULT2A1 which have been previously implicated in human drug and xenobiotic metabolism were cloned and expressed in *E. coli*. All the above isoforms except SULT2A1 were expressed as untagged proteins. Due to the difficulty associated with obtaining soluble expression of untagged SULT2A1, it was decide to express SULT2A1 as a fusion with maltose binding protein (MBP) in an attempt to improve its solubility. Fusion of insoluble proteins to MBP has been known to improve their solubility on expression in bacteria (Esposito and Chatterjee, 2006; Hayhurst, 2000; Kapust and Waugh, 1999). Kinetic analysis was also performed on the recombinant isoforms in addition to substrate specificity profiling for each of the recombinant isoforms generated. In order to see if a correlation exists between the functional properties and structural features of recombinant bovine SULTs, primary sequence alignment with human SULTs was carried out using ClustalW. To see the effect of differences in primary sequence on the overall protein structures, tools such as the Discovery Studio Visualizer 3.1 (DS visualizer)

(http://accelrys.com/products/discovery-studio/visualization-download.php) were used to analyse the crystal structures of the relevant human and mouse SULTs.

3.2 Expression of recombinant bovine sulfotransferases

Untagged bSULT1A1, bSULT1B1, bSULT1E1 and bSULT2A1 were cloned into the expression vector pET17b and expressed in *E. Coli.* All the untagged bSULTs were expressed in the soluble fraction except for bSULT2A1 whose presence was detected in the total cell lysate but not in the soluble fraction of the cell suggesting it was present in inclusion bodies. Hence it was decided to expresses bSULT2A1 as a fusion with maltose binding protein (MBP). Expression as an MBP fusion resulted in the soluble expression of bSULT2A1, however, no activity in the MBP fusion or recombinant bSULT2A1 was detected against known SULT2A1 substrates such as DHEA, androsterone and pregnenolone. All the other isoforms expressed untagged and in the soluble fraction were found to be active towards known substrates for their human orthologs. Shown in figure 12 is an SDS-PAGE gel containing uninduced and induced bovine SULT isoforms.

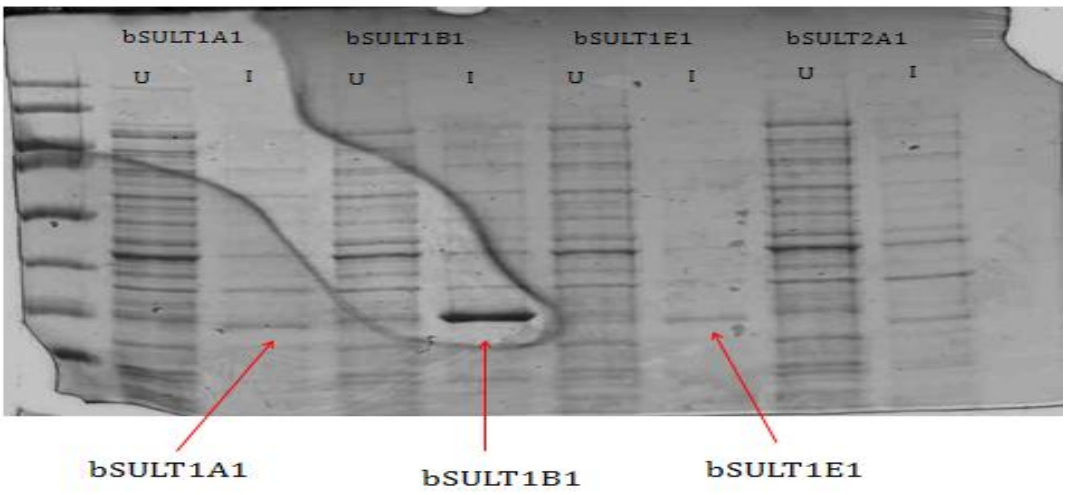

Figure 12: Expression of recombinant bovine SULTs

Bovine SULTs before (uninduced, U) and after (Induced, I) induction with IPTG. 20µg of protein was loaded in every well. Molecular weight markers were loaded in the first well on the left hand side.

Given below are western blots showing detection of recombinant bovine SULTs using anti human SULT antibodies.

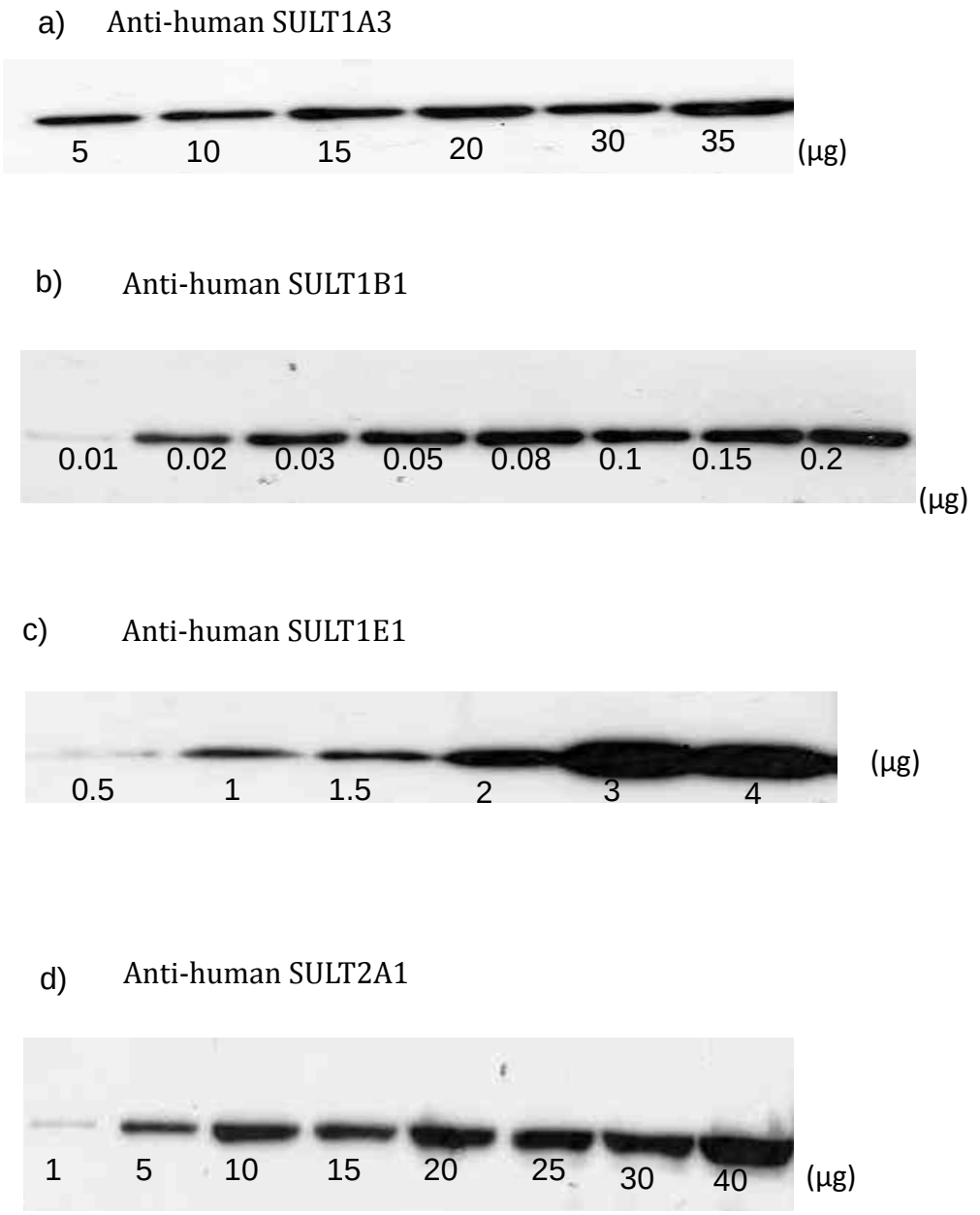

Figure 13: Detection of bovine SULTs using anti human SULT antibodies.

Anti-human SULT1A3 (1:5000) was used in the detection of bSULT1A1 (a), anti-human SULT1B1 (1:1000) MAP peptide antibody used in the detection of bSULT1B1 (b), anti-human SULT1E1 (1:10,000) used in the detection of bSULT1E1 (c) and anti-human SULT2A1 (1:10,000) used in the detection of bSULT2A1 expressed as a MBP fusion protein (d).

3.3 Analysis of sequence identity between bovine and human SULT isoforms

Due to the lack of availability of antibodies against bovine SULTs, it was decided to use human SULT antibodies. Anti-human SULT1A3 was used in the detection of bSULT1A1. It was raised against purified recombinant human SULT1A3 in sheep (Richard et al., 2001). Anti-human SULT1A3 has been used previously in the detection of both human SULT1A1 and human SULT1A3 (Riches et al., 2009). Anti-human SULT2A1 and anti-human SULT1E1 raised against purified recombinant human SULT2A1 and human SULT1E1, respectively, in sheep were used in the detection of bovine SULT2A1 and bovine SULT1E1. To determine if these antibodies would bind successfully to human SULTs, sequence alignment of whole protein was carried out between major human SULTs and their corresponding bovine isoforms. Results revealed a sequence identity ranging from 60-80% with SULT1B1 having the highest identity percentage of 81 whereas the SULT1E1 enzymes were the least identical at 62% (table 25). Although the bovine SULT isoforms were highly identical to their respective human SULTs, they were only 40-60% identical to each other. Sequence alignment analysis carried out to assess identity between different isoforms of bovine SULTs revealed that members of the same bovine SULT family were more identical to each other as compared to members of different families. SULT2A1 and SULT2B1 had the highest identity at 57% whereas SULT1A1 and SULT2A1 had the lowest at 40% (table 26).

SULT isoform	Amino acid sequence identity (%)
SULT1A1	78
SULT1B1	81
SULT1E1	62
SULT2A1	70

Table 25: Sequence identity between bovine SULTs and their human counterparts.

Analysis was carried out using ClustalW2

SULT sequence number	Bovine SULT	SULT sequence number	Bovine SULT	Sequence similarity score
1	SULT1A1	2	SULT2A1	40
1	SULT1A1	3	SULT1E1	44
1	SULT1A1	4	SULT1B1	54
1	SULT1A1	5	SULT2B1	54
2	SULT2A1	3	SULT1E1	49
2	SULT2A1	4	SULT1B1	44
2	SULT2A1	5	SULT2B1	57
3	SULT1E1	4	SULT1B1	56
3	SULT1E1	5	SULT2B1	48
4	SULT1B1	5	SULT2B1	47

Table 26: Sequence identity between isoforms of bovine sulfotransferases.

Analysis was carried out using ClustalW2.

A multiple antigenic peptide antibody (MAP) directed towards a 17 amino acid sequence unique to human SULT1B1 and sharing very low identity to other human SULTs has been previously used in the detection of human SULT1B1. The antigenic agent consists of many copies of the 17 amino acid sequence attached to a poly-lysine chain which increases the size of the antigenic peptide so that it becomes big enough to elicit an immune response(Riches et al., 2009). In order to use the human SULT1B1 MAP antibody for the detection of bovine SULT1B1, it has to have high sequence identity with bovine SULT1B1 and have a very low

identity with other bovine SULT isoforms. Sequence alignment analysis was carried out on the MAP peptide sequences from human SULT1B1 and bovine SULT1B1 revealed a 76% identity between the two (see table 27). Human SULT1B1 MAP peptide shared less than 30% identity with the rest of the bovine SULTs. Like the human SULT1B1 MAP peptide, the bovine sequence corresponding to the human SULT1B1 sequence used for synthesis of the MAP shared low identity (<25%) with rest of the bovine SULT isoforms.

a)

```
bSULT1B1        MTSP-KDVLRKNLKLIHG 17
bSULT1A1        MELI-QDTSRPPAKYVKG 17
bSULT1E1        MSSS-KPSFSDYFGKLGG 17
bSULT4A1        MAES-EAETPSTPGEFES 17
bSULT2B1        MGEPAEPRNQAKWDPYE- 17
bSULT2A1        MTGKFLWFEGIPFPSVD- 17
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b)

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                *
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SULT	Amino acid sequence corresponding to the human SULT1B1 MAP peptide	Sequence identity score (%) relative to human SULT1B1_MAP	Sequence identity score (%) relative to bovine SULT1B1_MAP
Human SULT1B1	MLSPKDILRKDLKLVHG	100	
Bovine SULT1B1	MTSPKDVLRKNLKLIHG	76	100
Bovine SULT1A1	MELIQDTSRPPAKYVKG	29	23
Bovine SULT1E1	MSSSKPSFSDYFGKLGG	23	23
Bovine SULT2A1	MTGKFLWFEGIPFPSVD	5	5
Bovine SULT2B1	MGEPAEPRNQAKWDPYE	11	11
Bovine SULT4A1	MAESEAETPSTPGEFES	5	5

Table 27: Summary of sequence alignment of MAP region of human SULT1B1 with the corresponding region in other bovine SULTs

Sequence alignment of MAP region of human SULT1B1 with the corresponding sequence in other bovine SULTs (a) carried out using Clustalw2. The bovine sequence had a 76% sequence identity to the human SULT1B1 sequence used for the synthesis of the MAP antibody. It however had a low sequence identity with the corresponding MAP region of other bovine SULTs. (b)

3.4 Cross-reactivity of anti-human SULT antibodies with bovine SULTs

In order to see if anti-human SULT antibodies cross reacted with their bovine counterparts, 5µg bSULT1A1, 0.1µg bSULT1B1, 1µg bSULT1E1 and 15µg MBP-bSULT2A1 were loaded on to each of 4 gels (figure 14). The gels were then blotted with anti-human SULT1A3, anti-human SULT1B1 (MAP peptide), anti-human SULT2A1 and anti-human SULT1E1 antibodies. Anti-human SULT1A3 and anti- human SULT2A1 were specific for their respective SULT isoforms. The anti-human SULT1B1 peptide antibody cross reacted with bSULT1B1 but also bound non specifically to other unrelated proteins on the membrane. The anti-human SULT1E1 antibody strongly cross reacted with bSULT1E1 but also reacted with bSULT1A1 and bSULT1B1.

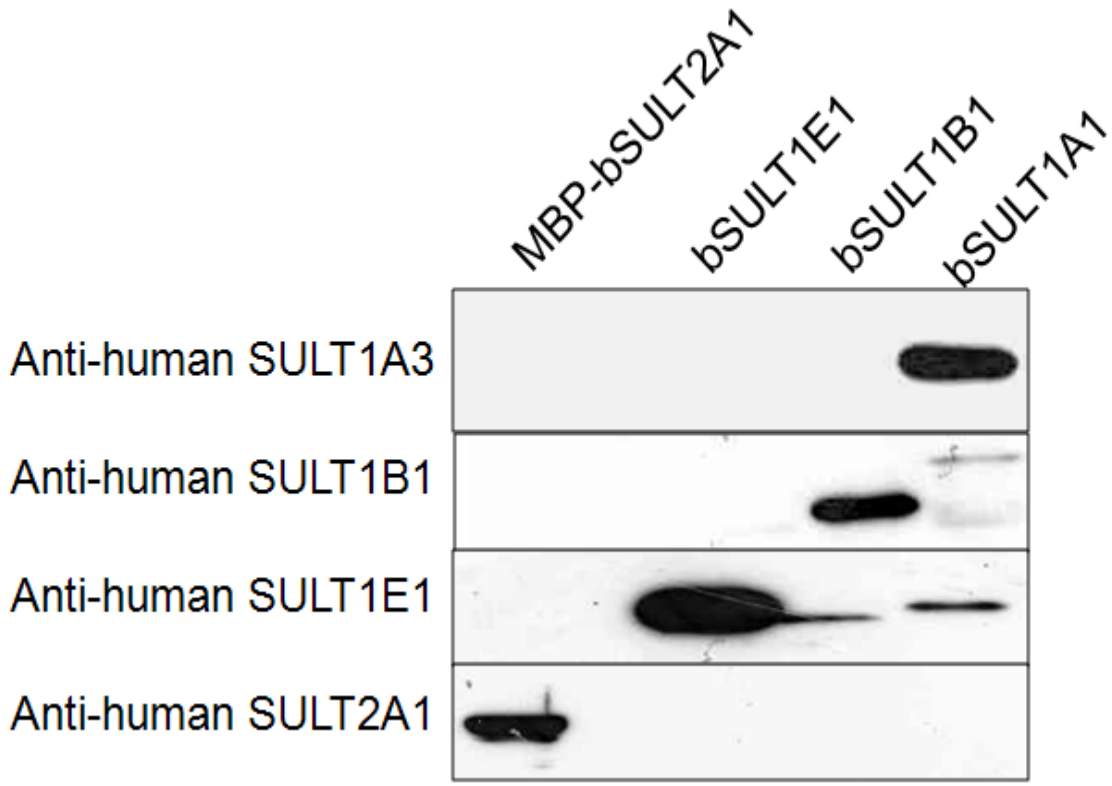


Figure 14: Detection of recombinant bovine SULTs in *E. Coli* cell free extract using anti human SULT antibodies.

5 µg bSULT1A1, 0.1µg of bSULT1B1, 1µg bSULT1E1 and 15µg of MBP-bSULT2A1 fusion was loaded on to every gel. Anti-human SULT1A3 cross reacts with bSULT1A1. Anti-human SULT1E1 cross reacts strongly with bSULT1E1 but also with bSULT1A1 and bSULT1B1

3.5 Optimization of conditions for enzyme kinetics with expressed recombinant bovine SULT isoforms.

SULT enzyme activity is affected by many factors such as protein concentration, concentration of the co-factor PAPS, incubation time, buffers used and pH of buffer used. Optimization for each of the above factors was done for recombinant bovine SULT enzymes, bSULT1A1, bSULT1B1 and bSULT1E1 used in this study. Protein concentration and incubation time were chosen such that they fell within the linear range of the reaction phase where increase in protein concentration

and incubation time would result in an increase in enzyme activity. Concentration of PAPS to be used was chosen such that it was not rate limiting. Enzyme activity is also heavily dependent on the pH of the buffer used. Biological buffers were made up in the physiological pH range from 5.2-9. Tris Maleate was used to buffer in the pH range of 5.2-5.6 Phosphate buffer, 5.8-8 and Tris HCl, 8-9. The buffer and pH in which highest enzyme activity was seen was chosen to be used. Table 28 shows an example of optimization with each of the above mentioned factors.

Recombinant bovine sulfotransferase	Substrate	pH	PAPS (µM)	Buffer	time (min)	protein (µg)
bSULT1A1	4-Nitrophenol	6.2	20	phosphate	20	20
bSULT1B1	4-Nitrophenol	6.2	20	phosphate	20	20
bSULT1E1	17β-estradiol	6.2	20	phosphate	20	30

Table 28: Summary of optimised conditions used for enzyme kinetics assays for recombinant SULT isoforms.

3.6 Bovine SULT1A1, SULT1B1 kinetics with 4-nitrophenol

Upon optimization of parameters affecting enzyme activity, kinetic analysis was performed on recombinant bSULT1A1 and bSULT1B1. Probe substrates of human SULTs were used for assessing the enzyme activity of the bovine counterpart. To date no known probe substrate has been identified that is exclusively metabolised by bSULT1B1. However, SULT1B1 is known to metabolise majority of the compounds turned over by bSULT1A1 (Fujita et al., 1999; Wang et al., 1998). 4-nitrophenol (4NP) was used as a substrate for assessing the activity of both bSULT1A1 and bSULT1B1. bSULT1A1 demonstrated partial substrate inhibition with 4-nitrophenol with a K_m of 33 µM and a K_i of 24µM (figure 15). A lower K_m of 4µM and a higher K_i of 34µM was recorded in a previous study for recombinant

purified human SULT1A1(Riches et al., 2007). However, it should be noted that recombinant human SULT1A1 used was purified whereas bovine SULT1A1 used here was not. Kinetic parameters for bSULT1A1 activity were calculated by fitting the enzyme curve to the partial substrate inhibition equation (refer to section 2.2.4 of Materials and Methods). V_{max} (3512 pmol/min/mg) and K_m (31μM) for bSULT1B1 (figure 16) were calculated by fitting the curve to Michaelis-Menten equation for enzyme kinetics (refer to section 2.2.4 of Materials and Methods).

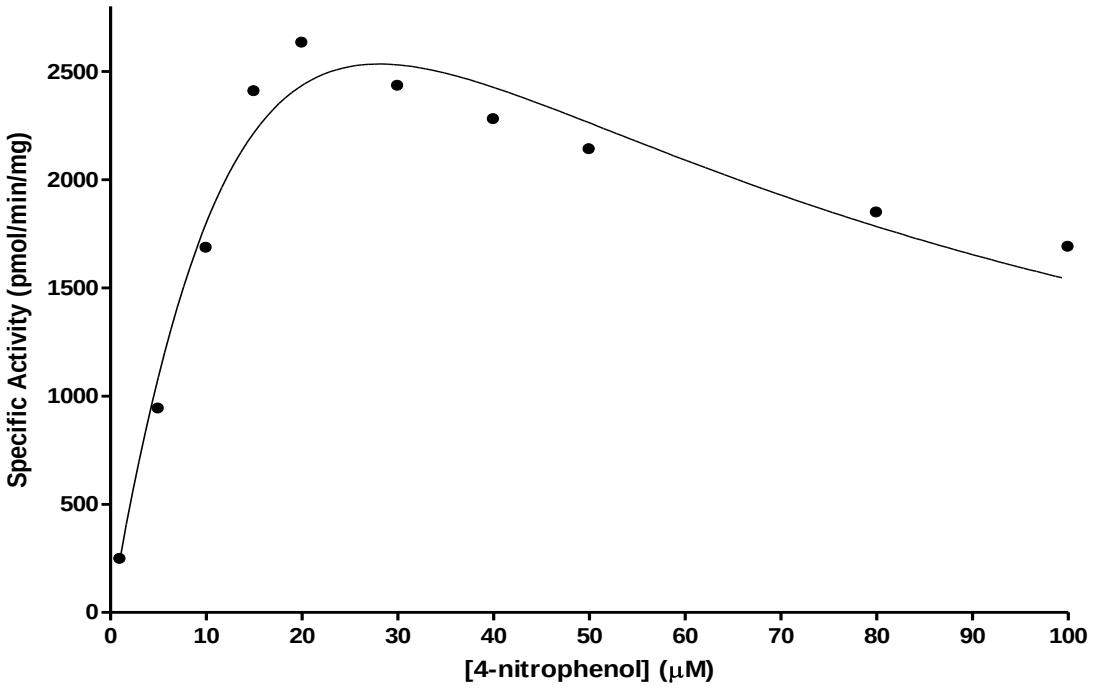


Figure 15: Rate of formation of 4-nitrophenol sulfate as a function of increasing 4-nitrophenol concentration (recombinant bSULT1A1).

[^{35}S] PAPS assay was used to determine sulfation of 4-nitrophenol. Each assay was carried out in duplicate. Data points are the average of the two values. Michaelis-Menten equation with partial substrate inhibition was used to analyse results as substrate inhibition was observed beyond 20μM 4-nitrophenol.

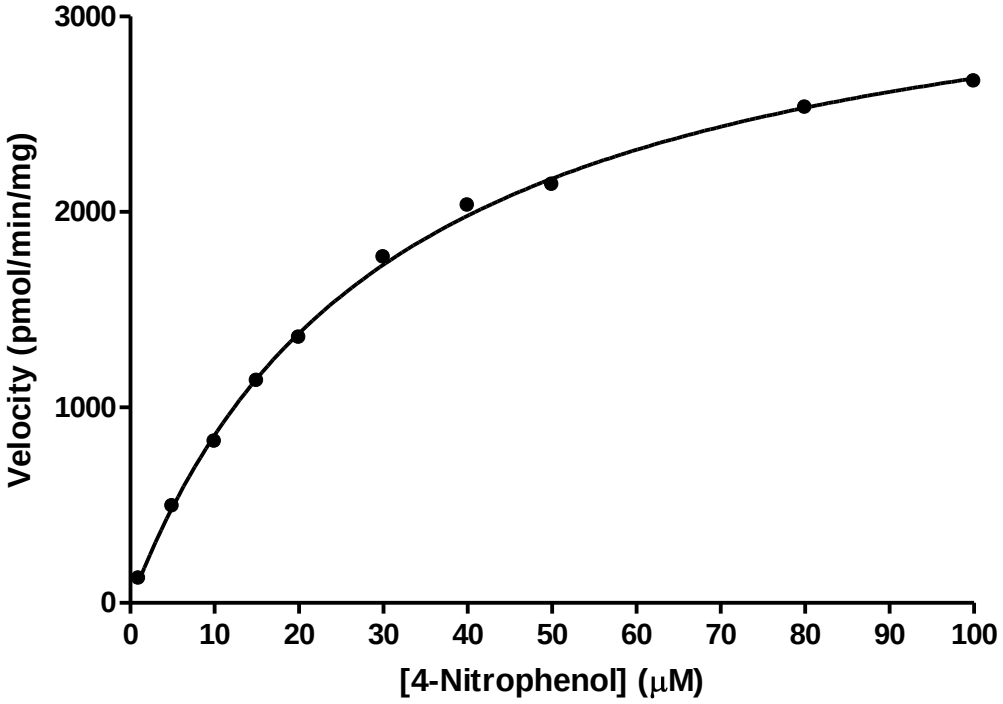


Figure 16: Rate of formation of 4-nitrophenol sulfate as a function of increasing 4-nitrophenol concentration (recombinant bSULT1B1)

[^{35}S] PAPS assay was used to determine sulfation of 4-nitrophenol. Each assay was carried out in duplicate. Data points are average of the two values. Michaelis-Menten equation was used to analyse results.

3.7 Substrate specificity profiling of recombinant bovine SULT1A1 and SULT1B1

In humans, both SULT1A1 and SULT1B1 are known to sulfate planar phenols with overlapping substrate specificities (Riches et al., 2007; Taskinen et al., 2003). In order to determine which of the two recombinant bovine SULTs was better at sulfation, a battery of phenolic compounds was screened for activity against recombinant bSULT1A1 and bSULT1B1. In order to make a valid comparison, it was necessary to do a substrate screen on equal amounts of recombinant bSULT1A1 (rbSULT1A1) and rbSULT1B1. This was achieved by loading

increasing amount of induced and uninduced bSULT1A1 and bSULT1B1 on the same gel (figure 17a) and using the software Quantiscan to measure the band density. The net band density volume was plotted against amount of induced protein loaded on to the gel. The experiment was repeated several times to ensure that the band densities fell within the linear range of the protein. The linear equation giving rise to the highest R^2 value was chosen (0.9912) to calculate the amount of bSULT1A1 present in the crude extract relative to the amount of bSULT1B1 (figure 17b). Using the equation, y=959.69x-7869.9 and 20µg of bSULT1B1 an equivalent of approximately 40µg bSULT1A1 protein of was calculated.

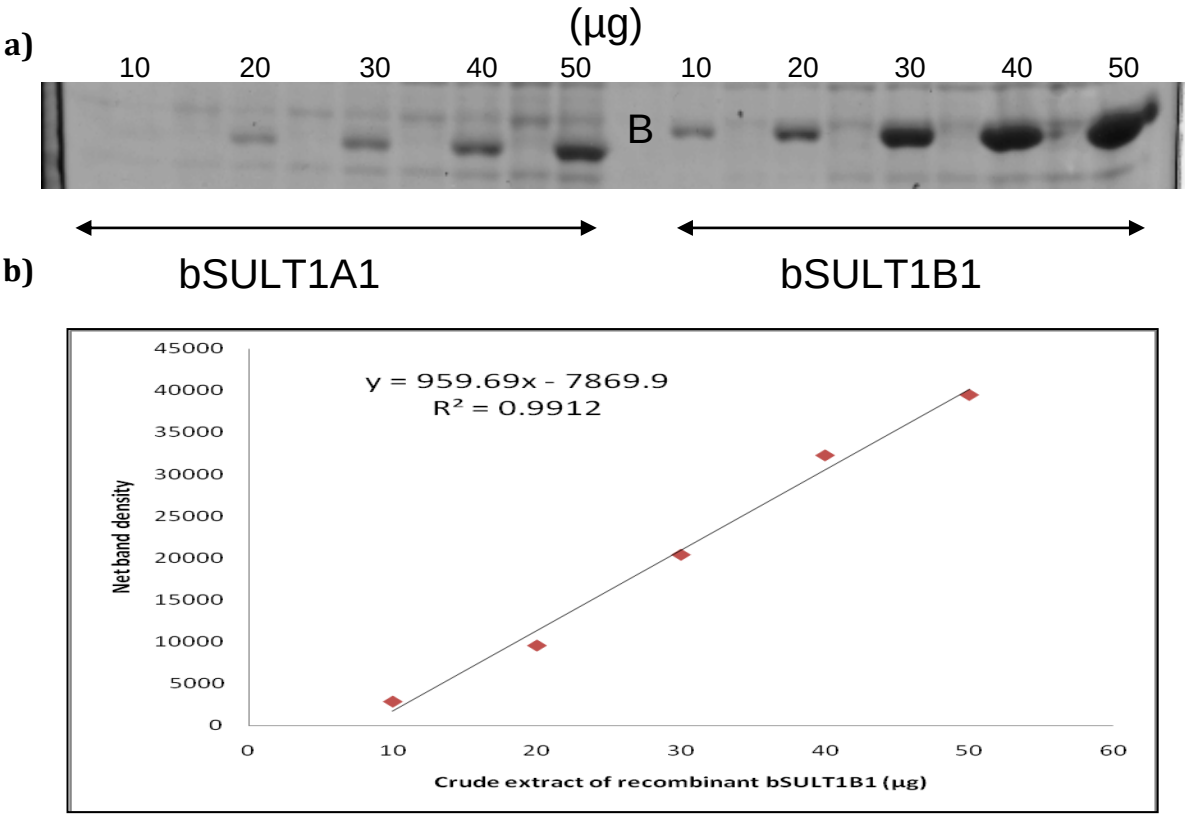
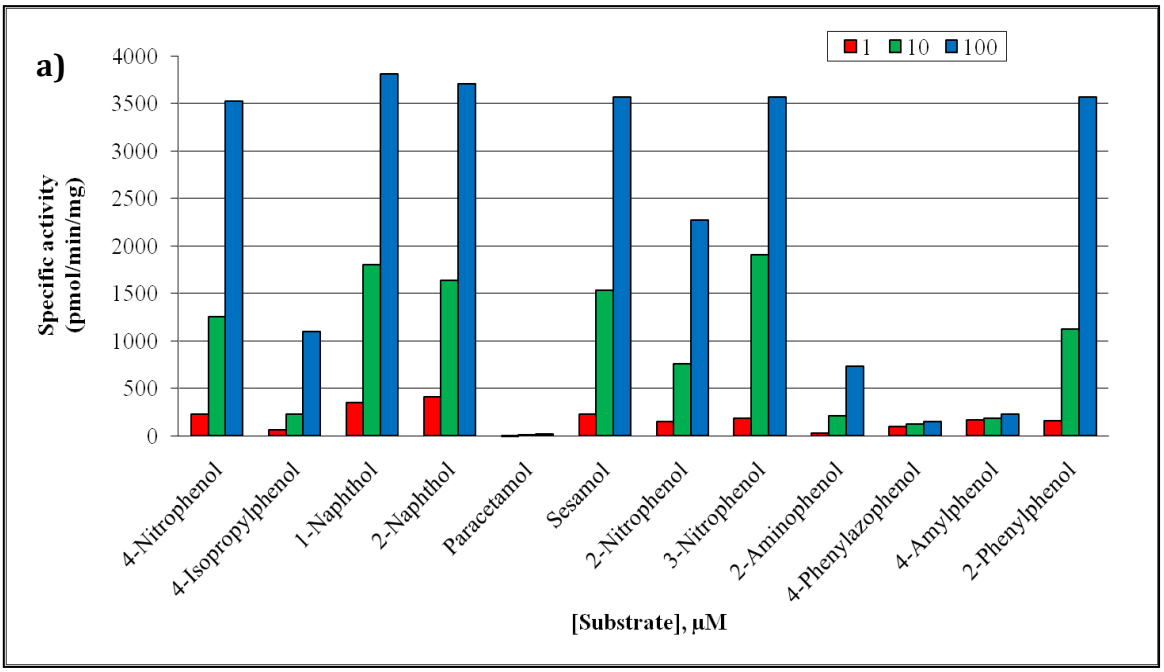


Figure 17: Calculation of amount of bovine SULT1A1 relative to bovine SULT1B1.

11% polyacrylamide SDS-PAGE gel containing 10-50µg of expressed recombinant bSULT1A1 and bSULT1B1 (a). The gel was scanned and imported into Quantiscan to obtain a net band density value. The band density values were plotted against the amount of protein for both bSULT1A1 and bSULT1B1. (b).

Enzyme activity was seen for bSULT1B1 with the majority of the tested compounds (figure 18). These were normally about twice that of bSULT1A1 indicating that bSULT1B1 was better at sulfation of phenolic compounds as compared to bSULT1A1. However, certain substrates such as 4-isopropylphenol, 2-aminophenol, 4-amylphenol and 4-phenylazophenol had higher activity with recombinant bSULT1A1. 2-aminophenol has already been established as a better probe substrate for human SULT1A1 since it does not undergo partial substrate inhibition kinetics like 4-nitrophenol with SULT1A1. It is more specific for human SULT1A1 and is metabolised to a lesser extent by SULT1B1. Unfortunately no potential probe substrates that are exclusively metabolised by SULT1B1 were detected. Paracetamol was the only substrate not metabolised by either enzyme.



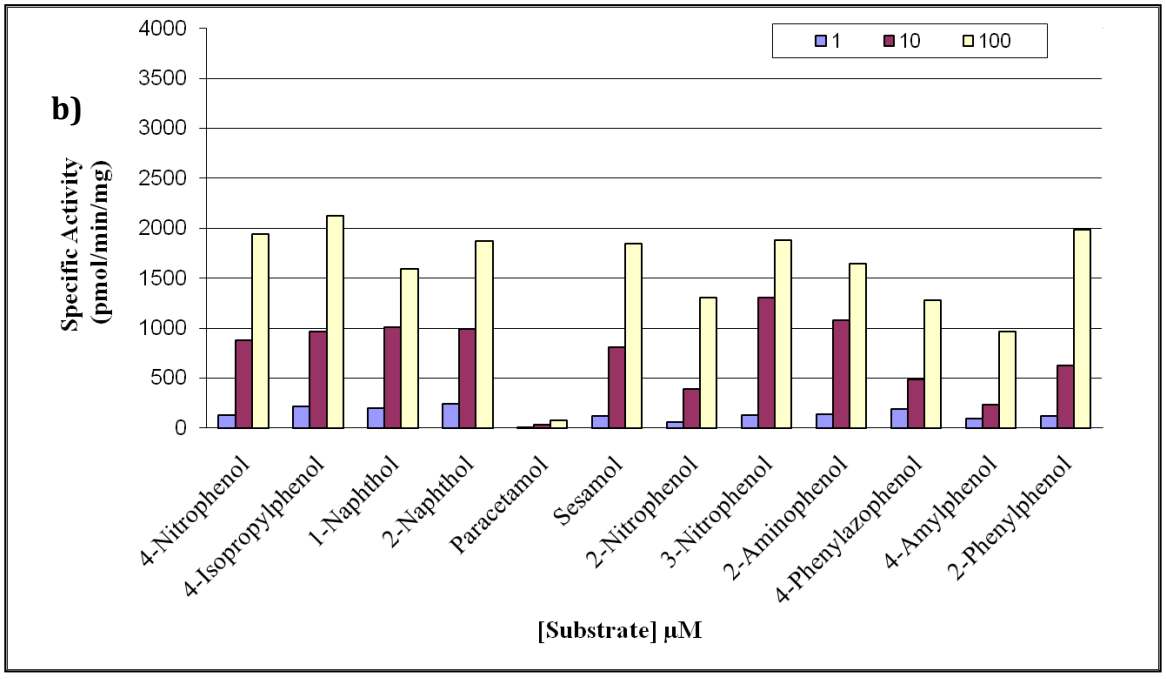


Figure 18: Substrate specificity profiling of recombinant bovine SULT1A1 and SULT1B1 with phenolic substrates.

A battery of phenolic substrates was screened for activity against 20µg of expressed recombinant bSULT1B1 (a) and 40µg of expressed recombinant bSULT1A1 (b) using the [^{35}S] PAPS assay. The substrates were used at 1, 10 and 100µM.

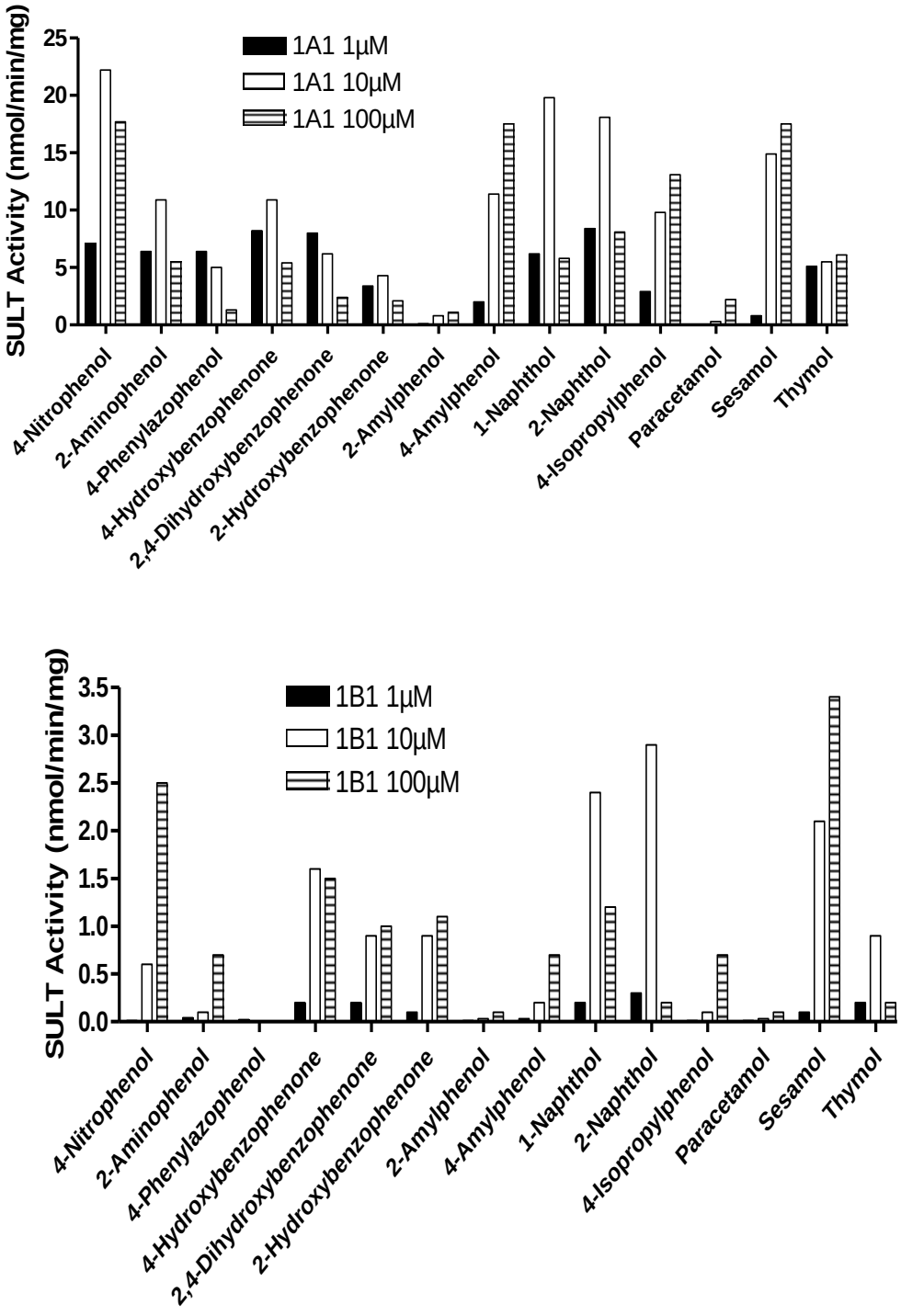


Figure 19: Substrate screen carried out on recombinant human SULT1A1 and SULT1B1 at 3 different concentrations (1, 10 and 100µM).

Sulfotransferase activity was measured with cell free extract expressing SULT1A1 (a) and SULT1B1 (b). Data are mean of experiments carried out in duplicate. This work was carried out by Zoe Riches and was published in Riches *et al* , 2007 (Riches et al., 2007). The graphs have been included in this thesis to aid comparison with recombinant bovine SULT1A1 and SULT1B1 data from figure 18.

3.8 Recombinant bovine SULT1E1 kinetics with 17β-estradiol and substrate specificity profiling with other xenobiotic substrates using [^{35}S] PAPS assay

In humans, 17β-estradiol is sulfated by SULT1E1 in the physiological (nanomolar) range. The recombinant purified human SULT1E1 has a K_m of 5nm and substrate inhibition is observed at concentrations above 80nM(Zhang et al., 1998). Expressed human SULT1E1 is capable of sulfating other steroids such as DHEA, pregnenolone, diethylstilbestrol and equilenin at micromolar substrate concentrations (Falany et al., 1995b; Falany et al., 1994). In addition to steroids, human SULT1E1 also has activity towards other xenobiotic compounds such as apomorphine, 4-*tert*-butyl-5-methoxycatechol, 3,4-dihyroxybenzoic acid ethyl ester and 4-isopropylcatechol (Taskinen et al., 2003). In this study, it was found that expressed recombinant bovine SULT1E1 metabolised 17β-estradiol in the micromolar range with a V_{max} of 1003 pmol/min/mg and a K_m of 2.3 μM. The enzyme demonstrated Michaelis-Menten kinetics and no substrate inhibition was observed (figure 20). To test the sulfation capacity of recombinant bovine SULT1E1 towards various other xenobiotic compounds, we screened a battery of substrates against bovine SULT1E1 using the [^{35}S] PAPS assay (figure 21). Substrates such as apomorphine and 4-isopropylcatechol known to have activity against recombinant human SULT1E1 were also used. A substrate concentration range of 0.01-10μM was chosen. Bovine SULT1E1 extensively metabolises 4-phenylphenol and does so in the micromolar range, 4-phenylazophenol, apomorphine and 4-isopropylcatechol are also metabolised. Sesamol was the only substrate not to be metabolised by recombinant bSULT1E1.

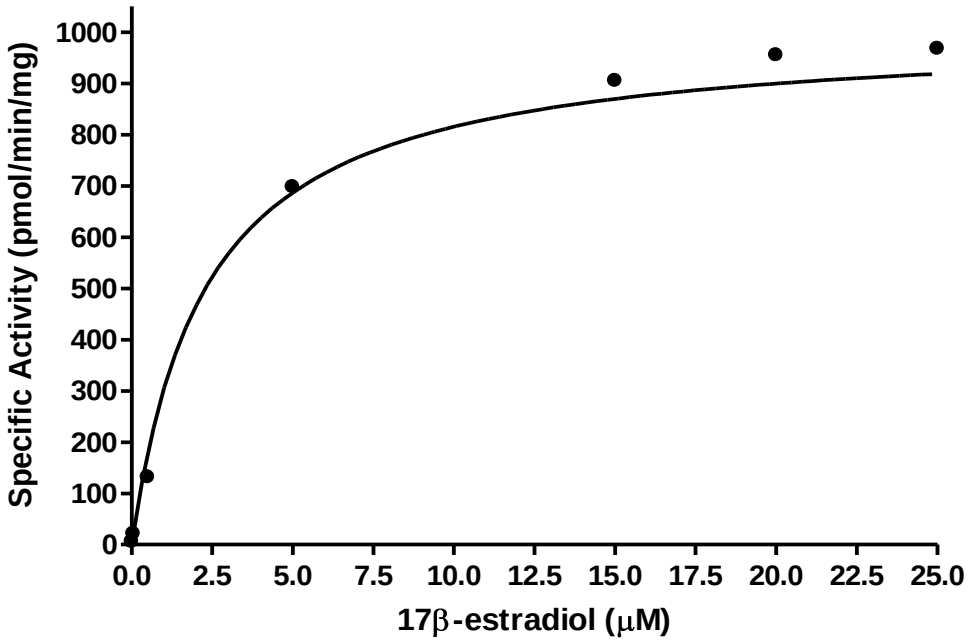


Figure 20: Rate of formation of 17β-estradiol sulfate as a function of increasing 17β-estradiol concentration.

[^{3}H] based solvent extraction assay was used to determine sulfation of 17β-estradiol. Each assay was carried out in duplicate. Data points are the average of the two values. Michaelis-Menten equation was used to analyse results.

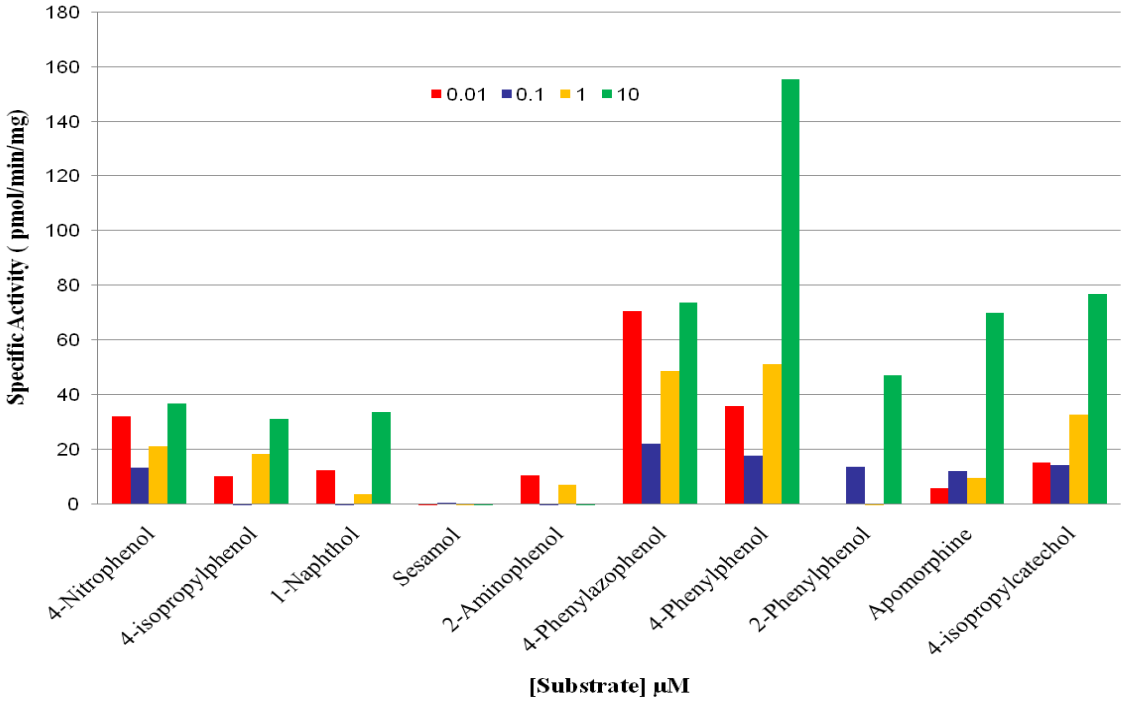


Figure 21: Substrate specificity profiling of recombinant bovine SULT1E1.

A battery of xenobiotic substrates was screened for activity against 30μg of expressed recombinant bSULT1E1 using the [^{35}S] PAPS assay. The substrates were used at 0.01, 0.1, 1, 10 μM.

Recombinant bovine SULT	Substrate tested	V_{max} (pmol/min/mg)	K_m (μM)	K_i (μM)
SULT1A1	4-nitrophenol	8494 ± 3812	33 ± 20	24 ± 14.5
SULT1B1	4-nitrophenol	3512 ± 55	31 ± 1.2	NA
SULT1E1	17β-estradiol	1003 ± 41.4	2.3 ± 0.6	NA
SULT2A1	DHEA and pregnenolone	ND	ND	ND

Table 29: Summary of recombinant bovine SULT kinetics.

Kinetic assays were performed on the given substrates using optimized conditions listed in table 3.4, the Michaelis-Menten equation was used to calculate Vmax and Km for all except SULT1A1 where partial substrate inhibition equation was applied. Each assay was carried out in duplicate. Values given above are ± standard error. ND refers to activity not detected and NA = Not applicable

3.9 Structural analysis of recombinant bovine sulfotransferase isoforms

Once expressed, radiolabelled enzyme assays were performed on recombinant bovine SULTs using substrates established for activity in humans and other species. It was discovered that the enzymatic properties of these enzymes differed markedly from those of the corresponding human SULTs. In order to understand the underlying structural aspects that could possibly govern some of these findings, it was decided to perform a structural analysis. This involved carrying out a primary amino acid sequence alignment of bovine SULTs with SULTs from humans and other species followed by a tertiary structure analysis to see the effect of primary amino acid changes on the overall structure.

3.9.1 Structure of bovine SULT1E1 compared to those of mouse Sult1e1 and human SULT1E1.

SULT1E1, more commonly known as estrogen sulfotransferase, has a very high affinity for the endogenous substrate 17β-estradiol which it sulfates in the low

nanomolar range(Forbes-Bamforth and Coughtrie, 1994). Multiple sequence alignment using ClustalW was carried out between SULT1E1 from human, mouse and cow (See figure 22). The conserved TYPKSGTTW motif (grey) known as the phosphosulfate binding loop (PSB), is involved in PAPS binding. The entire motif including the critical lysine residue at position 48 (yellow) is absolutely conserved in SULT1E1 from the 3 species. The KXXXTVXXXE motif (264-273, human SULT1E1 numbering) plays an important role in the dimerization of SULTs. Human SULT1E1 exists as a dimer in solution in contrast to mouse SULT1E1 which is a monomer(Kakuta et al., 1997). The 'TV' in this motif is replaced by a 'PE' in mouse SULT1E1. It appears that bovine SULT1E1 might exist as a dimer in solution since the dimerization motif of human and bovine SULT1E1 are identical. Critical residues that are conserved and involved in substrate binding of SULT1E1 have been highlighted in green. His108 is absolutely conserved in all SULTs and mutation of this residue abolishes SULT activity. Residues that are involved in substrate binding but are not very well conserved are highlighted in blue. Phe231 (human SULT1E1 numbering) in the substrate binding pocket of SULT1E1 is replaced by a Leucine (figure 24). Since phenylalanine contains an aromatic ring it confers more hydrophobicity to the highly non polar substrate binding pocket by forming greater van der Waals forces with the surrounding residues. Substitution of an aromatic residue like phenylalanine to an aliphatic leucine means lesser van der Waals interactions that reduce the overall hydrophobic nature of the binding pocket which in turn could affect substrate binding.

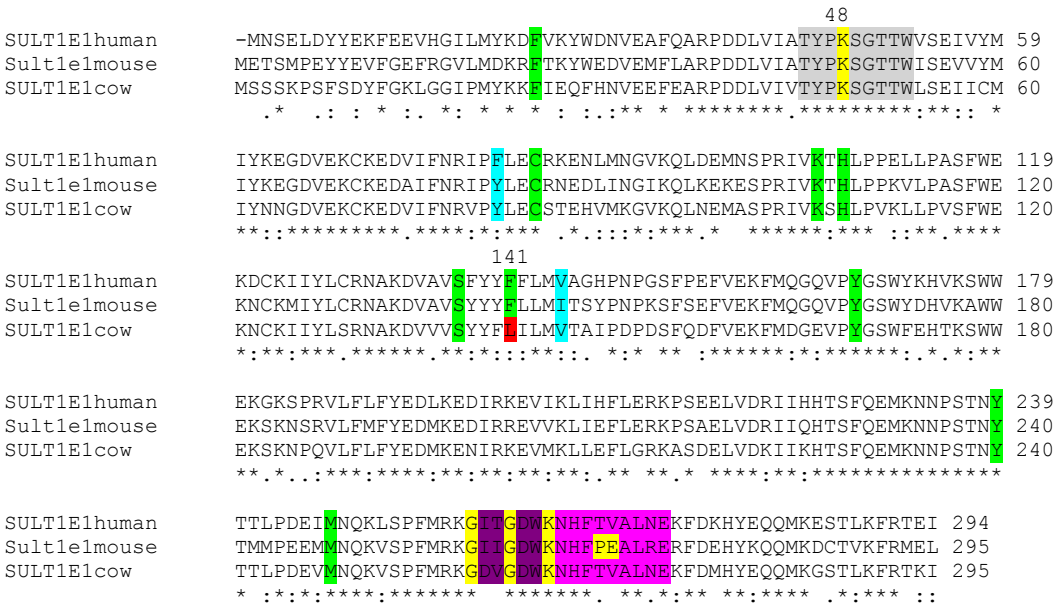


Figure 22: Multiple sequence alignment of SULT1E1 from human mouse and cow.

The highlighted regions show important structural features. Residues 45-53 form the PSB loop which is involved in binding PAPS. The region highlighted in pink is the KXXXTVXXXE motif that is involved in the dimerization of the protein. Upstream of this motif is the well conserved GXXGXXK motif that also plays a role in PAPS binding. Residues highlighted in green are conserved in the active site whilst those highlighted in blue are residues in the active site which are not very well conserved. Phe141 is conserved in human and mouse but not in the cow.

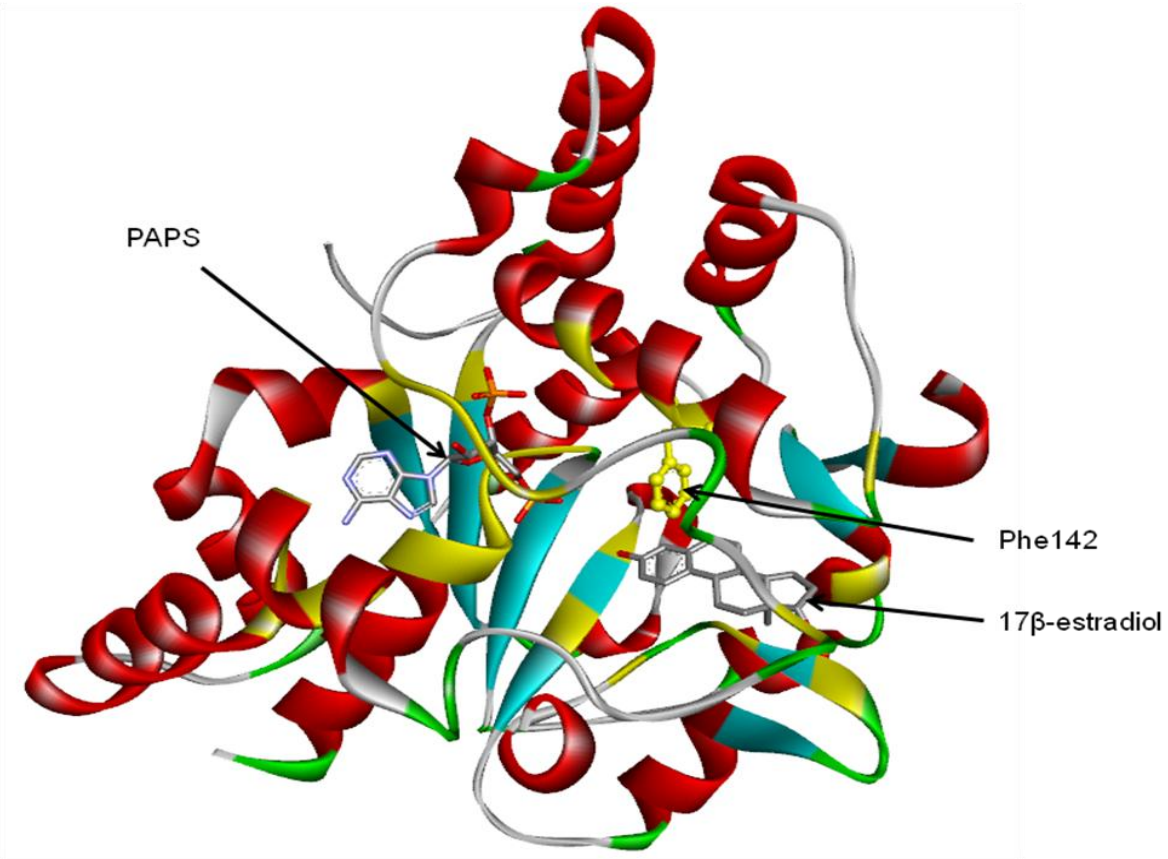


Figure 23: Crystal structure of mouse Sult1e1 complexed with the co factor PAPS and the substrate 17β-estradiol.

The figure has been downloaded from protein data bank. www.pdb.org ID:1AQU. The alpha helices are shown as red ribbons and β-sheets as turquoise strands. The active site of the protein is highlighted in yellow. Also shown in yellow is a ball and stick model of Phe142 which is substituted by a leucine in bovine SULT1E1(Kakuta et al., 1997)

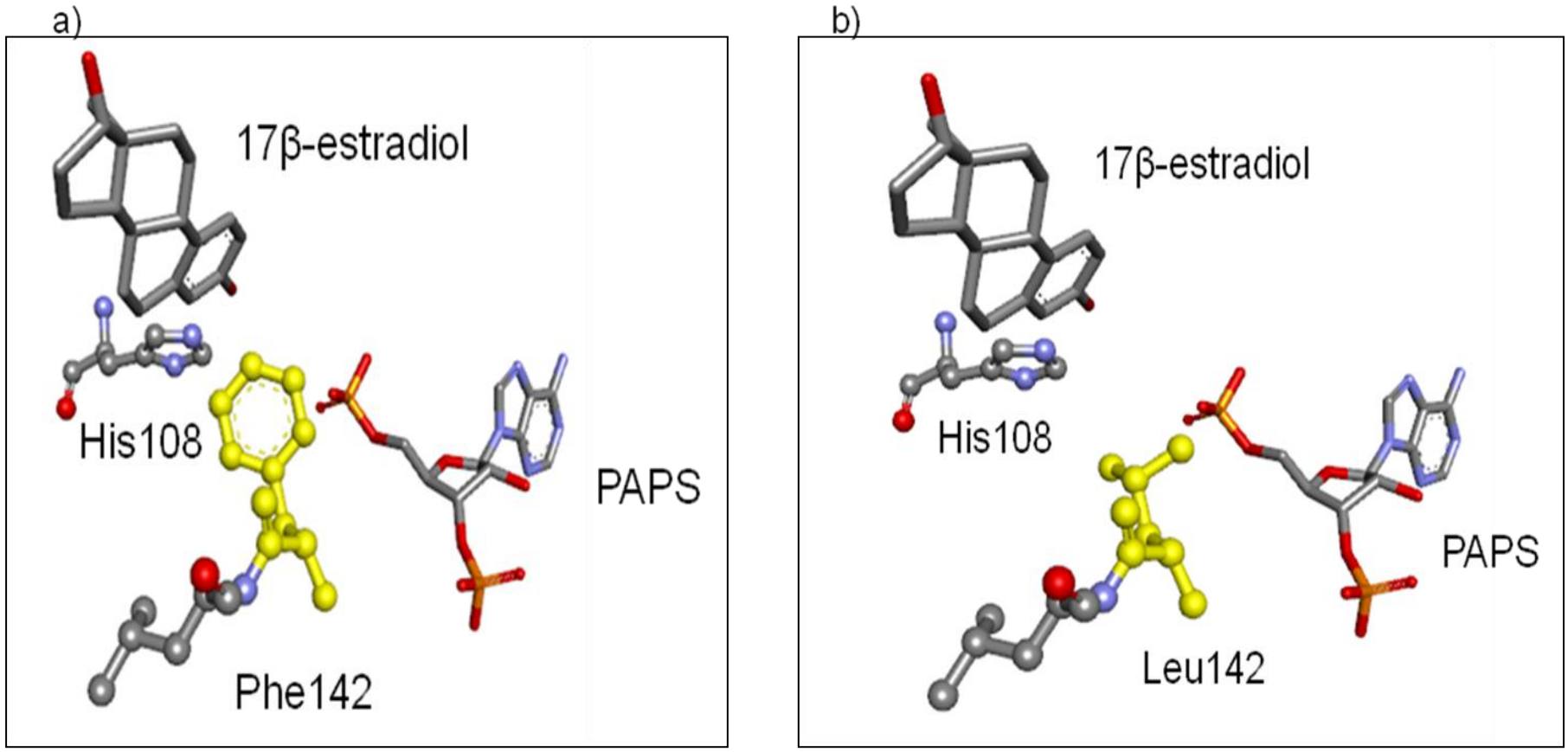


Figure 24: Positions for the co-factor PAPS, the substrate 17β-estradiol and the residue Phe142/Leu142 in the active site of mouse Sult1e1/bovine SULT1E1.

The file for visualising the crystal structure was downloaded from www.pdb.org , 1AQU(Kakuta et al., 1997). For the purpose of clarity the remainder of the protein is not shown here. In mouse Sult1e1, Phe142 (yellow) is in a position to form van der Waals interactions with 17β-estradiol (a). Phe 142 is substituted for Leu 142 (yellow) in bovine SULT1E1 and a depiction of this substitution is shown above in the active site of mouse Sult1e1 (b). This substitution could affect the binding of 17β-estradiol. His108 is a critical residue in the active site.

Polychlorinated biphenyls (PCBs) are environmental pollutants found in terrestrial and aquatic systems. They interfere with sexual development and reproduction by exerting their endocrine disrupting effects. It was found that some hydroxylated PCBs (OH-PCBs) can inhibit SULT1E1 by binding to it in a position similar to that of 17β-estradiol. We identified that Phe141 in human SULT1E1 is in a critical position in the active site of the enzyme. The aromatic hydrocarbon ring of phenylalanine is in a position to form stronger van der Waals interactions with the substrate than compared to Leu141 in bovine SULT1E1. This substitution could affect the binding of the inhibitor as well as 17β-estradiol (see figure 25 and 26).

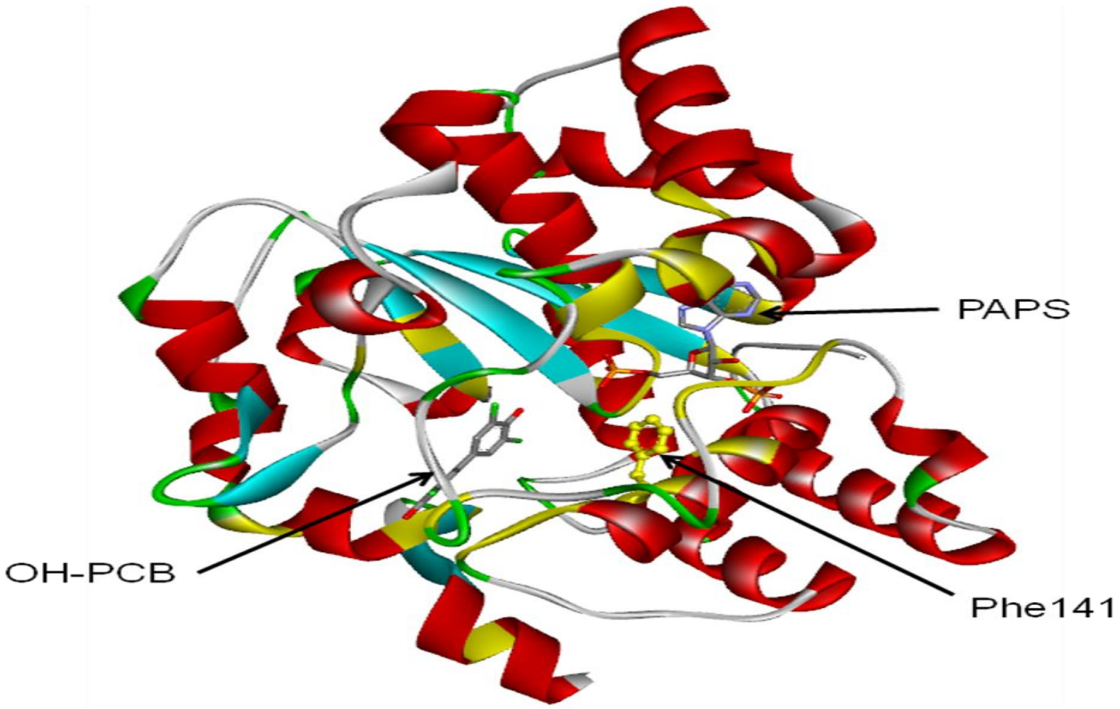


Figure 25: Crystal structure of human SULT1E1 complexed with the co factor PAPS and the inhibitor 4,4-OH 3,5,3',5'-tetra CB (OH-PCB).

The inhibitor OH-PCB binds in the active site of human SULT1E1 in a position similar to that of 17β-estradiol. The data file view to generate this figure has been downloaded from protein data bank. www.pdb.org ID:1G3M(Shevtsov et al., 2003). The alpha helices are shown as red ribbons and β-sheets as turquoise strands. The active site of the protein is highlighted in yellow. Also shown in yellow is a ball and stick model of Phe141 which is substituted by a Leucine in bovine SULT1E1

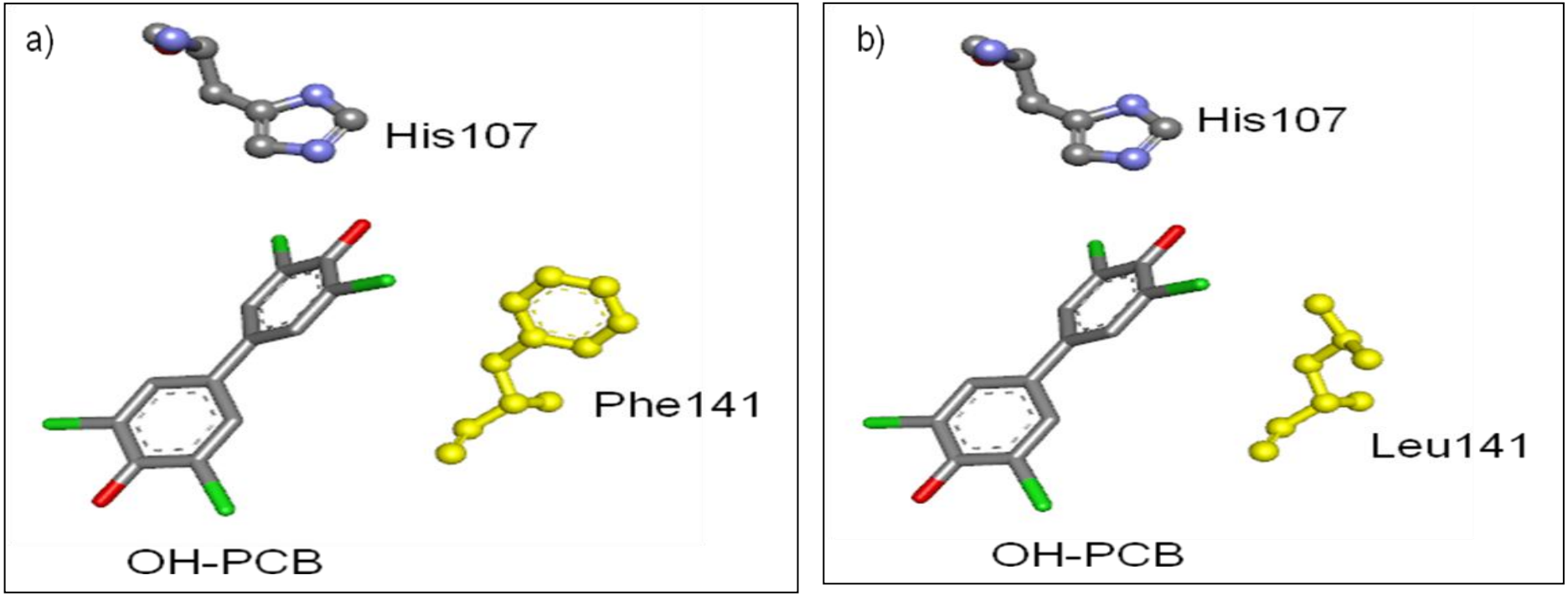


Figure 26: Positions for the co-factor PAPS, the inhibitor OH-PCB and the residues Phe141/Leu141 in the active site of human SULT1E1.

The data file view to generate this figure was downloaded from www.pdb.org , 1G3M(Shevtsov et al., 2003). For the purpose of clarity the remainder of the protein is not shown here. Phe 141 (a) is substituted for Leu 141 (b) (yellow) in bovine SULT1E1 and a representation of this substitution is shown above in the human SULT1E1 active site. His107 is a critical residue in the active site.

3.9.2 Primary and tertiary structure of bovine SULT2A1 compared to that of human SULT2A1 complexed with the co-factor PAP and the substrate lithocholic acid

SULT2A1 is known as the bile acid sulfotransferase due to its ability to sulfate bile acids. In addition to bile acids, it also sulfates steroids such as DHEA and androsterone. Sequence alignment using ClustalW was carried out between SULT2A1 from human and cow (figure 27). Important motifs involved in PAPS binding and dimerizations were conserved. Conserved critical residues involved in substrate binding have been highlighted in green. Residues that were critical to substrate binding in human SULT2A1 but are not conserved in bovine SULT2A1 are shown in blue. In human SULT2A1, region 231-253 has been identified as the substrate binding pocket. Sequence analysis results revealed that majority of the residues in this region were not conserved between human and bovine. These included Tyr231, Ser235, Val240, Asp241, Lys242, Ala243, Glu244 and Ser251 (figure 28 and 29). Some of these changes were not considered very significant because the substituted amino acid had similar properties i.e. Asp241Glu both have side chains that are positively charged. However, changes such as Ser235Lys and Val240Glu were thought to be more significant since the substituted amino acid was a charged one. This has the potential to disturb the hydrophobic nature of the pocket. Tyr231 is an important residue in the active site of human SULT2A1. It has been known to play a role in PAPS binding, dimerization and stabilization of the enzyme substrate complex (Rehse et al., 2002). Substitution of this residue with phenylalanine in the cow could mean loss of the above mentioned functions. Indeed, this could possibly be the reason why we were unable to express recombinant bovine SULT2A1. A deeper analysis of this region

was carried out using the structural tool DS visualizer to look closely at the active site of human SULT2A1 and compare it to that of bovine SULT2A1.

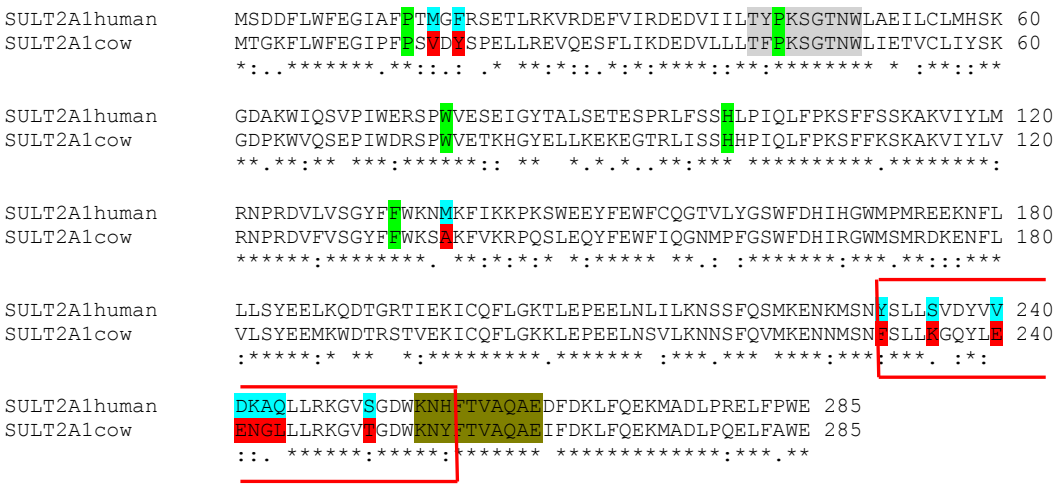


Figure 27: Multiple sequence alignment of SULT2A1 from human and bovine.

The highlighted regions show important structural features. Residues 45-53 form the PBS loop which is involved in binding PAPS. Region highlighted in dark green is the KXXXTVXXXE motif that is involved in the dimerization of the protein. Upstream of this motif is the well conserved GXXGXXK motif that also plays a role in PAPS binding. Residues highlighted in green are conserved in the active site whilst those highlighted in blue and red are residues in the active site which are not conserved. Region 231-253 shown in red box constitutes the substrate binding site. The region is not very well conserved between human and bovine.

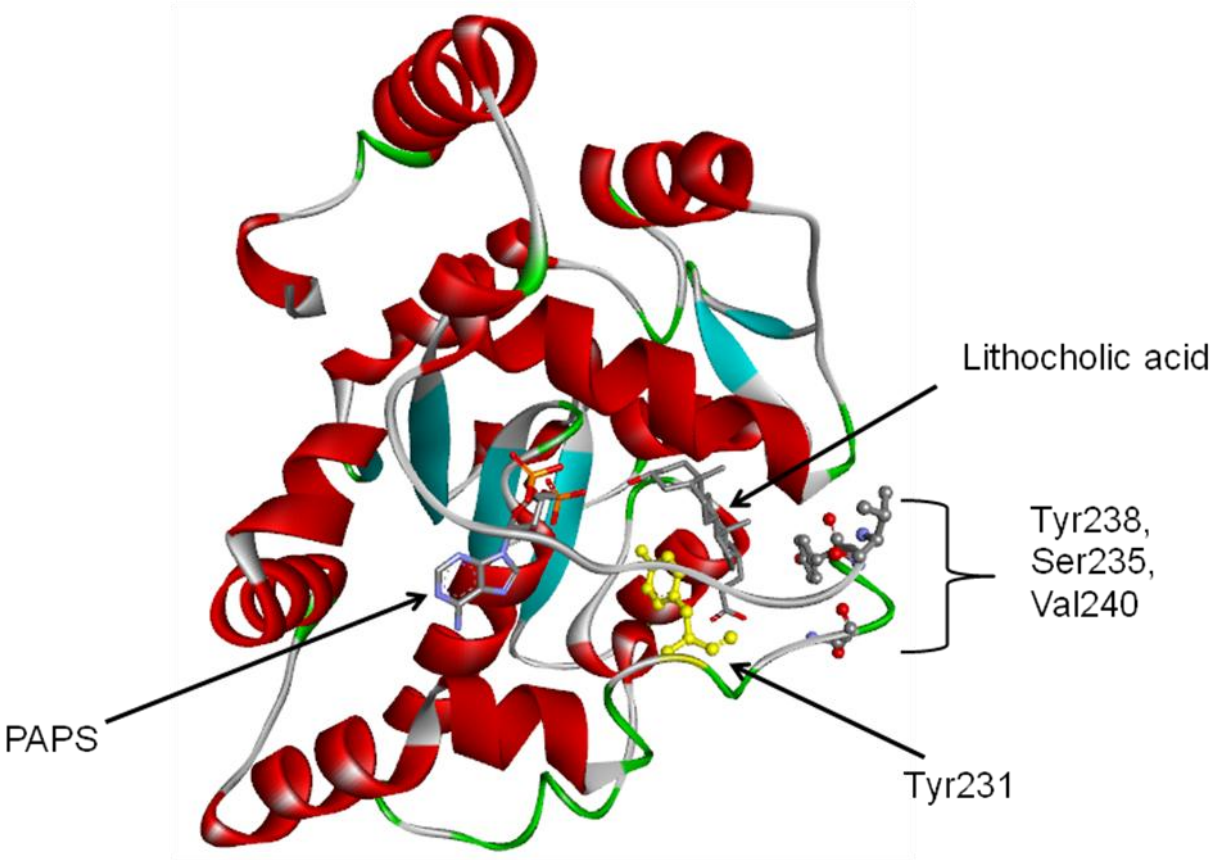


Figure 28: Crystal structure of human SULT2A1 complexed with the co-factor PAPS and the bile acid lithocholic acid.

Lithocholic acid binds in the active site of human SUL2A1 in a position similar to that of dehydroepiandrosterone (DHEA). The data file to generate this figure has been downloaded from protein data bank. www.pdb.org ID:3F3Y (To be published). The alpha helices are shown as red ribbons and β-sheets as turquoise strands. Shown in yellow is a ball and stick model of Tyr 231 which is substituted by a phenylalanine in bovine SULT2A1. Also shown is the ball and stick model of other amino acids in the substrate binding pocket such as Tyr238, Ser235 and Val240.

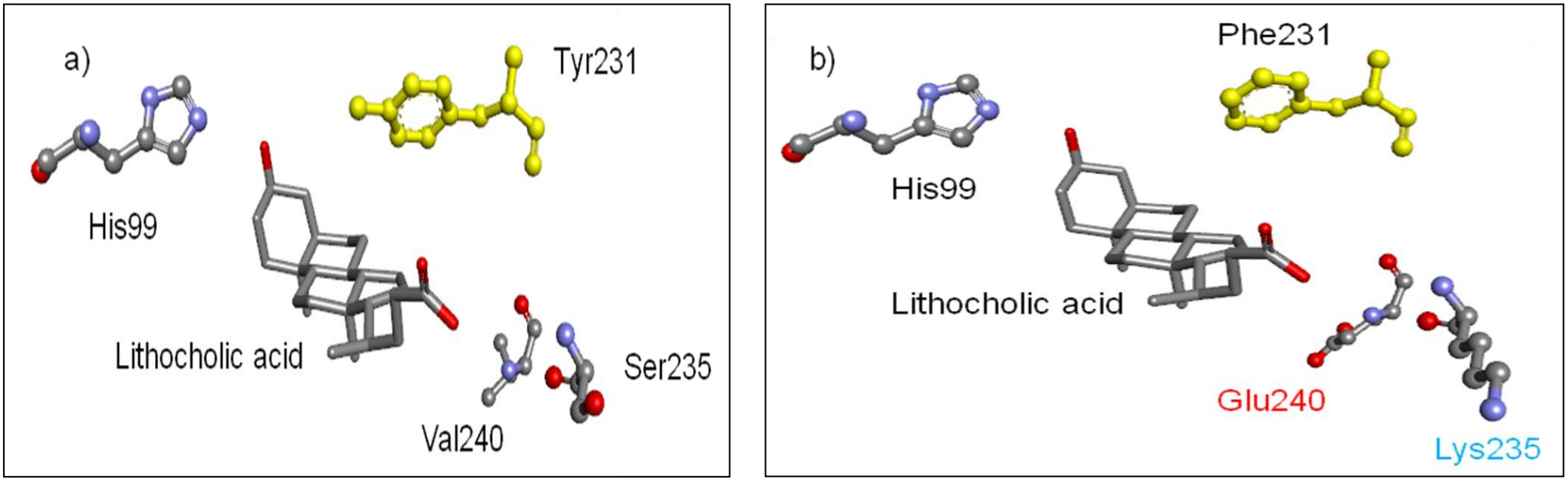


Figure 29: Effect of the substitution of critical residues in the active site of human SULT2A1.

Positions for the co-factor PAPS, the substrate lithocholic acid and residues Tyr231, Tyr238, Ser235 and Val240 in the active site of human SULT2A1 (a). The data file to generate this figure was downloaded from www.pdb.org , 3F3Y (To be published). For the purpose of clarity, the remainder of the protein is not shown here. In the amino acid sequence of bovine SULT2A1, the following substitutions were seen, Tyr231Phe, Ser235Lys, and Val240Glu. The effect of these substitutions is shown above on the active site of human SULT2A1 (b). His99 is a critical residue in the active site.

3.9.3 Primary and tertiary structure of bovine SULT1A1 compared to that of human SULT1A1 complexed with the co-factor PAP and two molecules of the substrate 4-nitrophenol

SULT1A1, the major human SULT, involved in drug metabolism, is known to metabolise a wide range of xenobiotic and endogenous compounds but specifically prefers small planar phenolic compounds. The crystal structure of human SULT1A1 solved in complex with the co-factor PAPS and the substrate 4-nitrophenol revealed that the enzyme accommodated not one but two molecules of 4-nitrophenol in its active site (Gamage et al., 2003). This was supported by kinetic data which showed that the enzyme exhibited substrate inhibition with 4-nitrophenol, a phenomenon whereby there is an impediment to catalysis when more than one molecule of the substrate binds to the active site. This is responsible for slowing the rate of reaction. It was also found that human SULT1A1 had a flexible substrate binding site that could adopt varying conformations to accommodate diverse substrates with varying sizes and shapes such as 17β-estradiol and diiodothyronine (Gamage et al., 2003). A sequence alignment was carried out between human and bovine SULT1A1 (figure 30). The PSB loop highlighted in grey was conserved. The KXXXTVXXXE motif highlighted in green was substituted for the KXXXSVXXXE motif in the bovine protein. Residues shown in blue are part of the active site which is involved in the binding of the first molecule of 4-nitrophenol (pNP^1) whereas those in yellow are involved in binding of the second molecule (pNP^2). Substitutions such as Ile89Val and Phe247Val in the active site of bovine SULT1A1 could affect the binding of pNP^2 (figure 31).

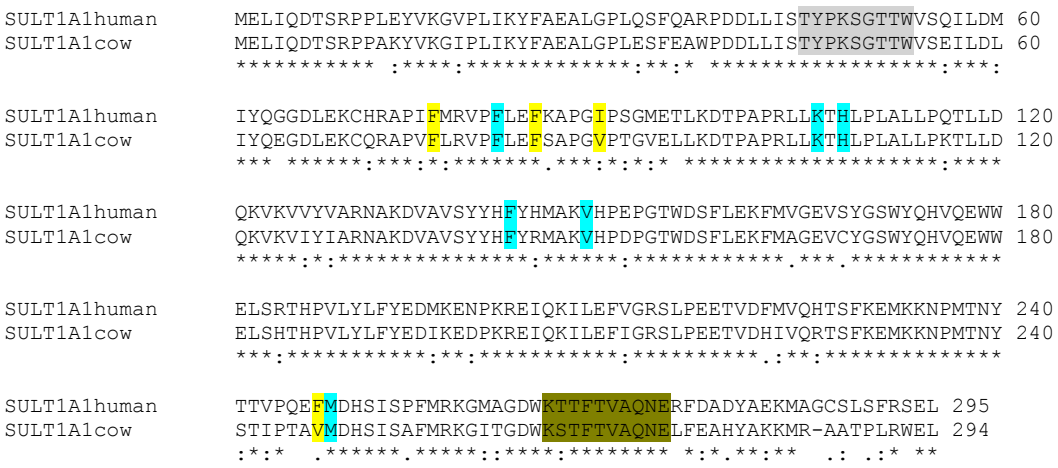


Figure 30: Multiple sequence alignment of human and bovine SULT1A1.

The highlighted regions show important structural features. Residues 45-53 form the PSB loop which is involved in binding PAPS (grey). The region highlighted in dark green is the KXXXTVXXXE motif that is involved in the dimerization of the protein. Upstream of this motif is the well conserved GXXGXXK motif that also plays a role in PAPS binding. Residues highlighted in blue are involved in binding one 4-nitrophenol (pNP[1]) whilst those highlighted in yellow are residues in the active site which bind to the other 4-nitrophenol (pNP[2]) molecule.

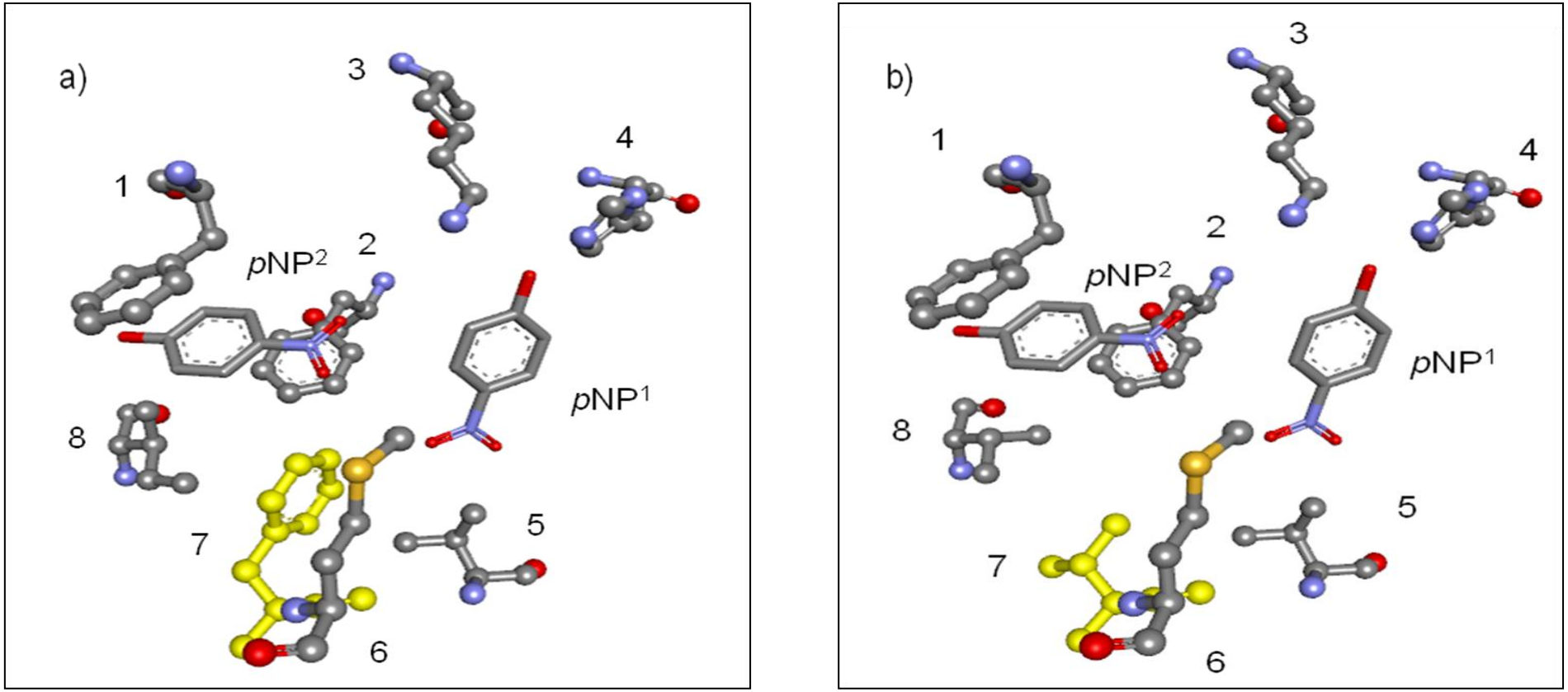


Figure 31: Effect of critical amino acid residue substitutions in the active site of human SULT1A1.

Shown above are the positions for the two 4-nitrophenol substrates and residues Phe76 (1), Phe84 (2), Lys106 (3), His108 (4), Val148 (5), Met248 (6) and Phe247 (7) and Ile89 (8) in the active site of human SULT1A1 (a). The crystal structure was downloaded from www.pdb.org , 3F3Y(Gamage et al., 2003). For the purpose of clarity the remainder of the protein is not shown here. In the amino acid sequence of bovine SULT2A1, Phe247 and Ile89 is replaced by valine. The effect of these substitutions is shown above on the active site of human SULT1A1 (b). His108 is a critical residue in the active site.

3.9.4 Primary structure of bovine SULT1B1compared to that of human SULT1B1

The human form of SULT1B1 was shown to be the major thyroid hormone sulfotransferase that has a higher affinity for tri-iodothyronine than SULT1A1. Along with thyroid hormone, the enzyme also metabolises other substrates such as tyrosine, dopamine and 4-nitrophenol. It has huge overlapping substrate specificity with SULT1A1. To date, no substrate exclusively metabolised by SULT1B1 has been discovere(Gamage et al., 2006). A sequence alignment analysis was carried out between human and bovine SULT1B1 (figure 32). Crystal structure of human SULT1B1 complexed with resveratrol was downloaded from protein data bank into the structural tool, DS visualizer (figure 33) and important residues in the active site were identified (highlighted in blue). All of them were found to be conserved between human and bovine.

```
SULT1B1human      MLSPKDILRKDLKLVHGYPMTCAFASNWEKIEQFHSRPDDIVIATYPKSGTTWVSEIIDM 60
SULT1B1cow        MTSPKDVLRKNLKLIHGCPITYAFANNWEKIEQFQSRPDDIMIVTYPKSGTTWISEIVDM 60
                  * ****:***:***:** *:* ***.********:******:*.*********:***:**

SULT1B1human      ILNDGDIEKCKRGFITEKVPMLEMTLPGLRTSGIEQLEKNPSPRIVKTHLPTDLLPKSFW 120
SULT1B1cow        VLHDGDVEKCKRDVITAKVPMLELALPGLRTSGLEQLEKNPSPRVVKTHLPIDLIPKSFW 120
                  :*:***:*****..** ******::*********:**********:****** **:*****

SULT1B1human      ENNCKMIYLARNAKDVSVSYYHFDLMNNLQPFPGTWEEYLEKFLTGKVAYGSWFTHVKNW 180
SULT1B1cow        ENNCKIIYLARNAKDVAVSFYHFDLMNNLQPLPGTWGEYLEKFLTGNVAYGSWFNHVKSW 180
                  *****:**********:**:***********:**** *********:*******.***.*

SULT1B1human      WKKKEEHPILFLYYEDMKENPKEEIKKIIRFLEKNLNDEILDRIIHHTSFEVMKDNPLVN 240
SULT1B1cow        WKKKEGHPILFLFYEDMKENPKQEIKKVVRFLEKNLDDEILDKIIYHTSFEMMKDNPLVN 240
                  ***** ******:*********:****::*******:*****:**:*****:********

SULT1B1human      YTHLPTTVMDHSKSPFMRKGTAGDWKNYFTVAQNEKFDAIYETEMSKTALQFRTEI 296
SULT1B1cow        YTHLPSEVMDHSKSSFMRKGIAGDWKNYFTVAQNEKFDAIYKKEMSETELQFRTEI 296

                  *****: *******.***** ********************:.***:* *******
```

Figure 32*:* Multiple sequence alignment of SULT1B1 from human and bovine.

The highlighted regions show important structural features. Residues 45-53 form the PBS loop which is involved in binding PAPS. Region highlighted in dark green is the KXXXTVXXXE motif that is involved in the dimerization of the protein. Upstream of this motif is the well conserved GXXGXXK motif that also plays a role in PAPS binding. Residues highlighted in blue are conserved in the active site of human and bovine SULT1B1.

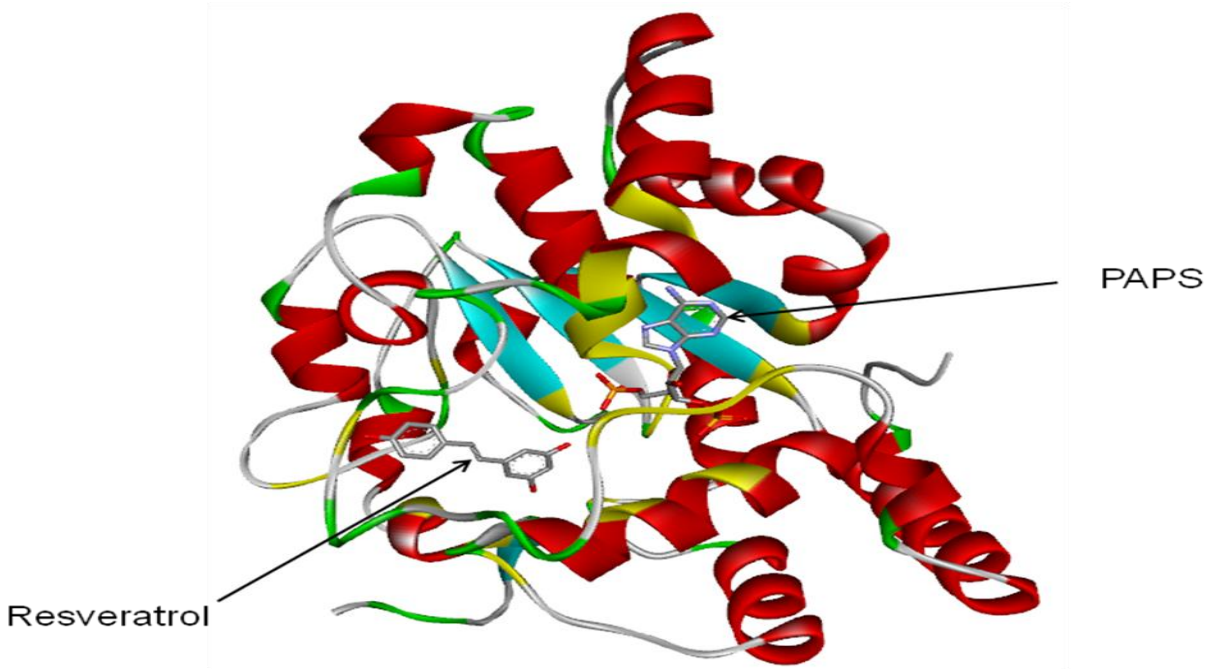


Figure 33: Crystal structure of human SULT1B1 complexed with the co factor PAPS and the substrate resveratrol.

The figure has been downloaded from protein data bank. www.pdb.org ID: 3CKL (To be published). The alpha helices are shown as red ribbons and β-sheets as turquoise strands. Shown in yellow are the residues present in the active site of the protein.

3.10 Discussion

In this chapter, bovine sulfotransferases were successfully cloned and expressed as recombinant proteins in an *E. Coli* expression system. Antibodies previously raised against human SULTs were used in the detection of the recombinant bovine SULTs. Amino acid sequence alignment of human and bovine SULTs was carried out to determine overall sequence identity and critical residues known to be involved in sulfation. Overall, a 60-80% sequence identity was noted between human SULTs and their corresponding bovine isoforms. Further to this, crystal structures of human SULT1A1, SULT1E1, SULT2A1, SULT1B1 and mouse Sult1e1 complexed with PAPS/substrate were downloaded from protein data bank and analysed to try and identify the structural implications critical residue modifications could have on the recombinant bovine sulfotransferases. Radiolabelled enzyme assays using substrates established for activity with human SULTs were used for carrying out functional studies on recombinant bovine SULTs. Thus an effort was made to establish a relationship between the structural and functional characteristics of some of the major sulfotransferases implicated in drug metabolism in humans and cattle.

Overall, the amount of bovine SULT immunoreactive protein detected using anti human SULT antibodies was less than the amount of human SULT immunoreactive protein detected using human SULT antibodies. Differences in primary amino acid sequence between human and bovine SULTs could mean that bovine SULT isoforms fold differently to that of human SULT isoforms such that anti human SULT antibodies do not bind as specifically to the bovine isoforms as they bind to the human ones. Results from structural analysis revealed that the PAPS binding loop (PBS) was conserved in all isoforms studied. The region known

to be involved in the dimerization of SULTs was also conserved in all except mouse Sult1e1 which is known to exist as a monomer in solution (Kakuta et al., 1997). The substrate binding region of human and bovine SULTs was the one that was the most variable. In SULT1E1, phenylalanine at position 141 in the active site of the protein is conserved in human and mice. However, in the bovine enzyme it is replaced by a leucine. Phenylalanine contains an aromatic ring that contributes to the hydrophobic nature of the active site. In mouse Sult1e1 and in human SULT1E1 it is in a position to form van der Waals interactions with the substrate. Substitution with a leucine decreases the overall hydrophobicity of the active site and it is highly possible that this could affect substrate binding. 17β-estradiol, the preferred substrate of SULT1E1 is usually sulfated in the low nanomolar range because of the high affinity that SULT1E1 has towards this substrate (Song and Melner, 2000). In this study, kinetic analysis of bovine SULT1E1 with 17β-estradiol revealed that the reaction had a K_m of 2.3μM and was predominantly sulfated in the micromolar range. This could quite possibly be the result of Phe141Leu substitution.

Of all the isoforms studied, SULT2A1 had the most variable substrate binding site. It was decided to study the following substitutions in detail as they were thought to be the most significant. Tyrosine at position 231 in human SULT2A1 is a very critical residue. In a substrate bound state this residue is involved in the stabilization of the enzyme substrate complex whereas in a non-bound state, the aromatic ring of Tyrosine orients itself away from the substrate binding region and plays a role in dimerization of SULT2A1(Rehse et al., 2002). It is also speculated that the hydroxyl group of tyrosine could form a hydrogen bond with the phosphate group of PAP, thus aiding the binding of PAPS (Rehse et al., 2002).

Replacement of tyrosine with phenylalanine means that there is no OH group available to interact with PAP. Also it remains to be established whether Phe231 can perform the other two roles of Tyr231. Apart from this, other substitutions in the active site of bovine SULT2A1 such as Ser235Lys and Val240Glu which introduce charged residues in the hydrophobic substrate binding pocket of bovine SULT2A1 could also have serious implications on the conformation of the active site and this in turn could affect substrate binding.

One of the characteristics of human SULT1A1 is that it displays extensive substrate inhibition with small planar phenolic compounds. The crystal structure of SULT1A1 complexed with 4-nitrophenol and PAPS showed that it binds 2 molecules of 4-nitrophenol (pNP^1 and pNP^2) in a catalytically competent manner in its active site and this is most likely responsible for substrate inhibition (Gamage et al., 2003). The hydroxyl group of pNP^1 is hydrogen bonded to the side chains of His108 and Lys106 whereas the nitro group forms van der Waals interactions with Val148, Phe247 and Met248. On the other hand pNP^2 binds weakly in to the active site with no interactions with the major catalytic residues. The aromatic ring of pNP^2 slots between the side chains of Phe84 and Phe76 whereas the nitro group forms van der Waals interactions with the side chains of Ile89 and Phe247 (Gamage et al., 2003). In bovine SULT1A1, Ile89 and Phe247 are substituted for valine. Valine although non polar like isoleucine and phenylalanine has a smaller and a non-aromatic side chain and will form lesser van der Waals interactions with pNP^2. This means that pNP^2 will not bind to the active site of bovine SULT1A1 as strongly as it binds to that of human SULT1A1. Substrate inhibition occurs when both pNP molecules bind to SULT1A1 and there is an impediment to catalysis. If pNP^2 binds weakly to bovine SULT1A1 compared

to human SULT1A1, the profound substrate inhibition seen with human SULT1A1 and 4-nitrophenol will not be observed with bovine SULT1A1. Indeed, kinetic analysis carried out in this chapter with recombinant bovine SULT1A1 using 4-nitrophenol as a substrate showed reduced substrate inhibition in comparison to its human counterpart.

The substrate binding site of bovine SULT1B1 was very well conserved with that of human SULT1B1. No major substitutions of critical residues in the active site were detected. Functional characteristics of recombinant bovine SULT1B1 assessed using 4-nitrophenol and a battery of other phenolic substrates was not very different (as compared to human SULT1A1 and bovine SULT1A1) to that of human SULT1B1 previously published (Riches et al., 2007) (figure 18 and 19).

In this chapter, strong correlation between the structural features and functional properties of bovine SULTs was established. Nevertheless in order to confirm our speculations with strong scientific evidence additional experiments need to be performed. For example side-directed mutagenesis of some of the above mentioned critical bovine SULT residues to their human counterpart followed by functional studies will prove useful in validating some of the above findings.

4. Characterisation of cytosolic Sulfotransferases in bovine liver

4.1 Introduction

The pharmaceutical industry has always relied on the extrapolation of pharmacokinetic data from laboratory animals to humans and other larger animals to predict drug toxicity (Smith, 1984). As our knowledge of drug metabolising enzymes and drug transporters increases, the complexity of interspecies differences becomes evident and making such extrapolations to predict *in vivo* drug toxicity has the potential to be seriously flawed. Extrapolation of drug metabolising capabilities between animals of different species is more complicated than extrapolation of pharmacokinetic data from animals to human. This is because of the wide variety of species out there with diverse anatomical and physiological attributes than humans (Lin, 1998). In order to minimize human health risk due to ADRs in livestock species it is essential to deliver safer drugs to animals of the livestock species. This can in part be achieved by having a thorough understanding of drug metabolism in animals of the livestock species. Since many drugs in humans and several other species are eliminated from the body as sulfate conjugates, it becomes very necessary to study drug metabolism brought about by SULTs. Knowing the amount of SULTs expressed in drug metabolizing organs is helpful as it allows commercial software tools such as Simcyp (www.simcyp.com) to incorporate the quantification data in their databases to predict the *in vivo* fate of drugs. Immunochemical quantification of CYPs and SULTs has been done in the past in human liver (Riches et al., 2009; Shimada T, 1994). Due to lack of bovine SULT specific antibodies it was not possible to immunoquantify SULTs in the bovine liver. Instead we made use of enzyme assays and existing SULT specific antibodies against other species including humans that cross reacted with bovine SULTs to identify major SULT isoforms present in bovine liver.

In the following series of experiments we have attempted to study the major drug metabolising isoforms of sulfotransferases. We focussed our attention on to SULT1A1, SULT1B1, SULT1E1 and SULT2A1. Using antibodies successfully developed against human SULT isoforms by previous lab members we detected expression of each of the above isoforms in male and female bovine liver. Progestins are synthetic progesterones that are used for synchronisation of estrus in cattle either alone or in combination with estrogen (Patterson et al., 1989). In humans SULT1E1 expression in the endometrium is tightly regulated under the influence of progesterones (Falany and Falany, 1996b). To investigate the effect of progestins on bovine SULT isoforms, a comparison of expression and activity was made between untreated female livers and female livers treated with an exogenous progestin*. Substrates established for activity with human SULT isoforms were used to determine activity of the 4 major drug metabolising SULTs in cattle. Conditions were optimized for pH, PAPS concentration, buffer, and incubation time and protein concentration in each liver set and with every substrate used. This was done to ensure that subsequent kinetics was performed at optimal conditions. Overall, an attempt was made to identify major SULT isoforms in bovine liver and relate their expression to activity in order to assess the validity of the substrates used.

4.2 Optimization of enzyme assay conditions

In order to carry out kinetics at optimal conditions, it is important to optimize assay conditions for parameters that could affect enzyme activity. pH, concentration of non-radiolabelled PAPS, choice of buffer, incubation time and protein concentration were optimised with 4-nitrophenol (SULT1A1, 1B1), 17β-

* Exogenous progestin: Identity of this drug is confidential to Pfizer.Inc

estradiol (SULT1E1) and pregnenolone (SULT2A1). Assay conditions in male bovine liver, female bovine liver (untreated) and female bovine liver (treated) were optimised separately with each substrate (Table 30) because of the potential for each of the 3 bovine liver set to have a different expression profile.

Bovine Liver cytosol	Substrate	pH	PAPS (µM)	Buffer	time (min)	protein (µg)
male	4-nitrophenol	5.8	20	phosphate	30	15
male	17β-estradiol	6.2	20	phosphate	30	30
male	pregnenolone	6.2	20	phosphate	30	30
female (UT)	4-nitrophenol	6.4	20	phosphate	15	30
female (UT)	17β-estradiol	6.2	20	phosphate	30	30
female (UT)	pregnenolone	6.2	20	phosphate	30	30
female (T)	4-nitrophenol	6.4	20	phosphate	15	30
female (T)	17β-estradiol	6.2	20	phosphate	30	30
female (T)	pregnenolone	6.2	20	phosphate	30	30

Table 30: Summary of optimised conditions used in bovine liver enzyme assays

Enzyme activity assays were optimised for pH, PAPS concentration, time protein and buffer for substrates listed above in male and female livers. UT stands for untreated female bovine liver and T for female bovine livers treated with an exogenous progestin. All male bovine livers used in this study were untreated.

4.3 Detection of expression of SULT2A1 in bovine liver using anti human SULT2A1 antibody

The hydroxysteroid sulfotransferase, SULT2A1 is commonly referred to as dehydroepiandrosterone (DHEA) sulfotransferase since it preferentially metabolises DHEA despite having a broad substrate predilection with other steroids such as pregnenolone, estradiol, estrone, testosterone and androsterone (Falany, 1997; Falany et al., 1995a; Falany et al., 1994). In some species such as the mouse and rabbit, SULT2A1 has a greater propensity for other steroids such

as pregnenolone(Kouichi Yoshinari, 1998; Shimizu et al., 2003), hence labelling SULT2A1 as a DHEA SULT would be very arbitrary.

SDS-PAGE gel electrophoresis was performed on 20 (male and female) bovine liver tissue cytosol samples of which 12 were female and 8 male. Of the 12 female bovine livers, 8 belonged to untreated females whereas the other 4 were from females treated with an exogenous progestin. All the 8 male livers were untreated. 8 untreated female and 8 male samples were analysed on the same gel (figure 34). Similarly, 8 untreated female and 4 treated female livers were also analysed on one gel (figure 36). This was done so that differences arising due to inter-gel or inter-blot variation can be minimised. Immunoblot analysis was carried out using anti human SULT2A1 antibody raised previously in our lab against purified recombinant human SULT2A1 (Richard et al., 2001). Blots were scanned using a standard desktop scanner (Hewlett Packard) and the software package QuantiScan32 (Biosoft) was used to calculate the densitometric volume of bands. This was performed in a similar manner to that described in chapter 3. SULT2A1 was detected in both male and female bovine liver. A considerable amount of variation was seen in the expression of SULT2A1 in between samples. In order to determine if the expression of bSULT2A1 in male livers was significantly different from the female livers a 2 tailed t-test assuming unequal variance was carried out (Table 31). The average net band density of male bovine SULT2A1 was almost twice that of female bovine liver SULT2A1. Statistical analysis (t-tests) results showed that there was a significant difference in expression of bSULT2A1 in the male and female liver. On the other hand no significant difference was observed in bSULT2A1 expression in females as a result

of treatment with an exogenous progestin. All the western blots shown here in this chapter were repaeated several times.

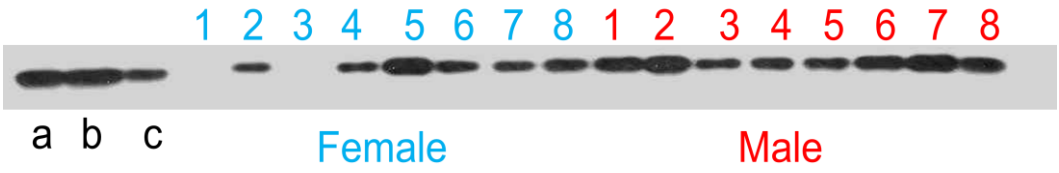


Figure 34: Bovine SULT2A1 expression in 8 untreated female and male livers.

30µg of liver cytosol was loaded for every liver sample. a, b and c contains 5, 3 and 1µg of recombinant bSULT2A1 respectively.

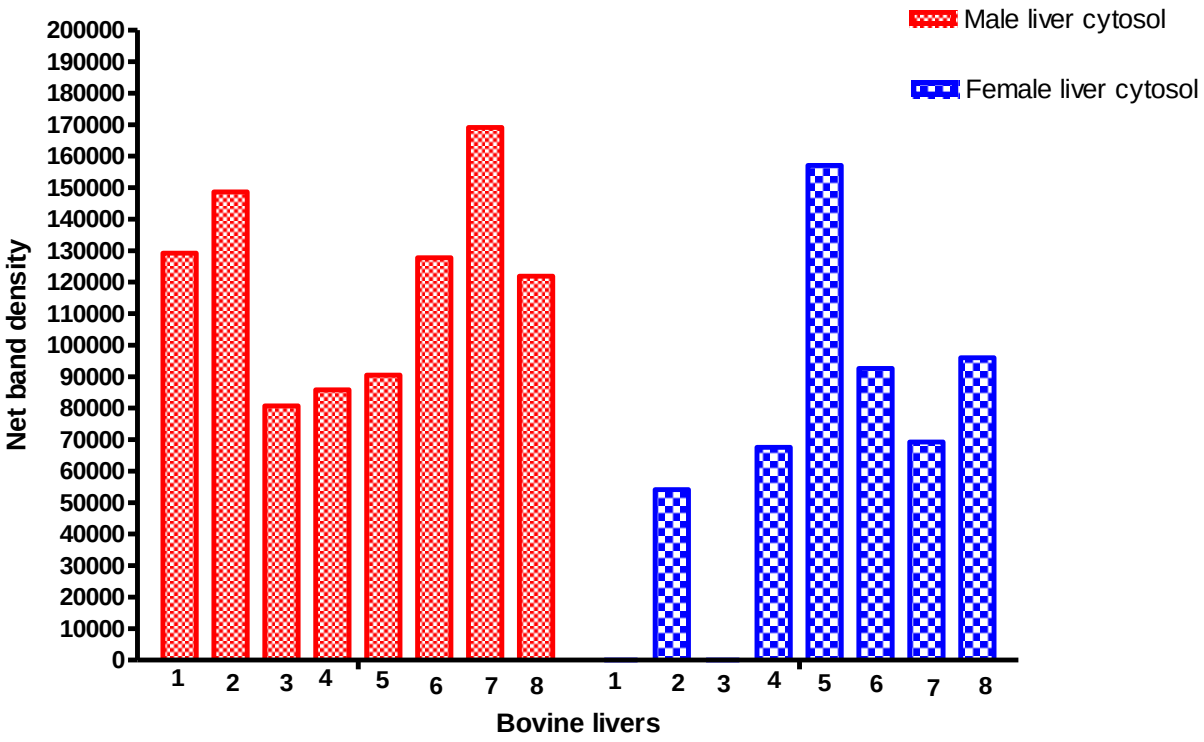


Figure 35: Net band density values as an indication of expression levels of bovine SULT2A1 in male and female bovine liver cytosol.

Net band density values were calculated by quantifying the band density of the scanned western blots and subtracting it from a set background value. For more information on this refer to chapter 3. Shown above are the net band density values (bovine SULT2A1 expression) for the 8 untreated bovine male and bovine female liver samples.

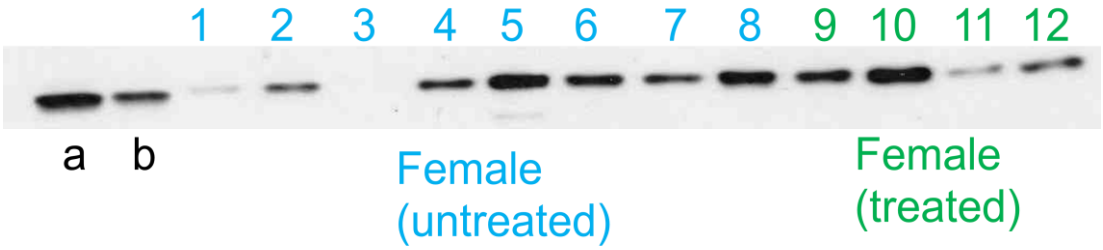


Figure 36: Bovine SULT2A1 expression in 8 untreated female and 4 treated female livers.

30µg of liver cytosol was loaded for every liver sample. a and b contains 3, 1 µg of recombinant bSULT2A1 respectively.

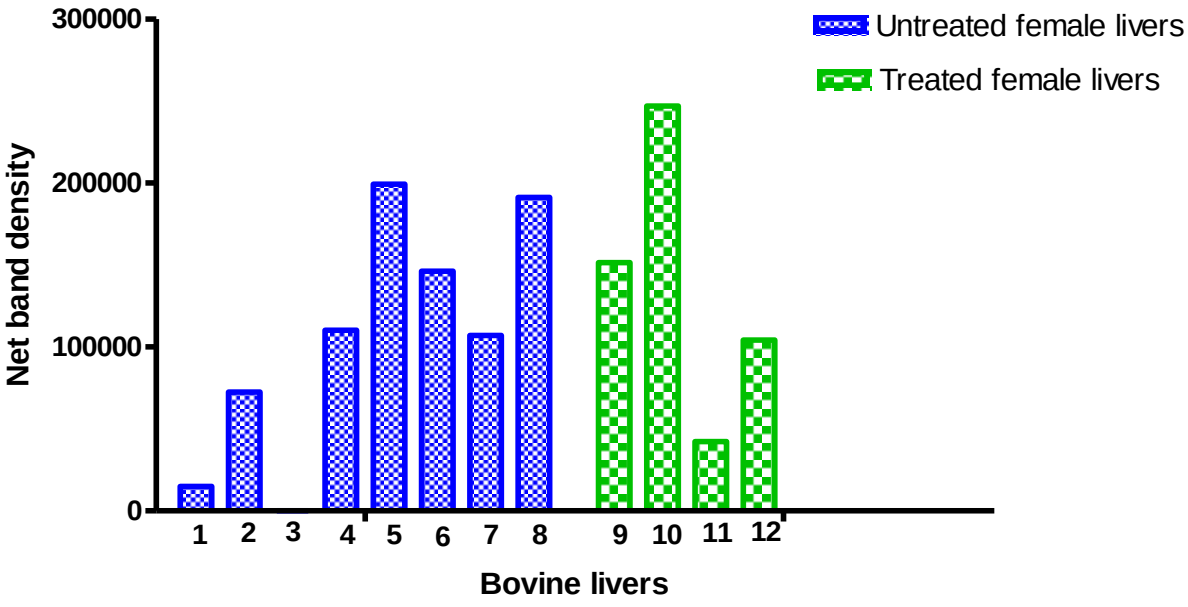


Figure 37: Net band density values as an indication of expression levels of bovine SULT2A1 in untreated and treated female bovine liver cytosol.

Shown above are the net band density values for bovine SULT2A1 expression in 8 untreated female (1-8) and 4 treated female (9-12) bovine liver samples.

Liver cytosol	No of samples (n)	Average net band densities	Standard deviation	P value	Result
Female (UT)	8	105200	73925	0.56	Null hypothesis accepted
Female (T)	4	136260	86285		
Male (UT)	8	119220	31550	0.03	Null hypothesis rejected
Female (UT)	8	67090	51775		

Table 31: Expression levels (average band densities) of bSULT2A1 in male and female bovine liver samples.

UT= Untreated and T= Treated. Null hypothesis (H_o) =There is no difference in expression levels of bSULT2A1 between male and female or between female (UT) and female (T). A two tailed t-test assuming unequal variance was carried out. Null hypothesis was rejected if P < 0.05

4.4 Analysis of bovine SULT activity towards pregnenolone in bovine liver samples

SULT2A1 specifically metabolizes dehydroepiandrosterone (DHEA) in humans and several other species. Using anti human SULT2A1 antibodies, SULT2A1 was detected in the bovine liver however; activity towards DHEA was not seen. Pregnenolone is metabolised by both human SULT2A1 and SULT2B1. It was decided to see if there was any activity towards pregnenolone in the bovine livers.

Kinetic studies were performed on pregnenolone sulfation in bovine livers using the optimized conditions listed in table 30. Substrate inhibition was observed in female bovine liver samples (figure 38 and 39) but not with male bovine liver samples (figure 40) which displayed Michaelis-Menten kinetics. V_{max} and K_m values were calculated for untreated female, treated female and male liver samples. No difference was seen in the V_{max} and K_m values between untreated and treated female livers (table 32) suggesting that probably the same set of enzyme

(s) were involved in pregnenolone metabolism in both. However, this cannot be said for pregnenolone metabolism in male bovine livers which had different kinetic properties to female bovine livers.

Liver	No of samples (n)	Vmax (pmol/min/mg)	K_m (µM)	K_i (µM)
Female (untreated)	8	39.8 ± 10.8	2.4 ± 1.2	12.4 ± 6.8
Female (treated)	4	46.1 ± 35.3	4 ± 4.7	6.9 ± 8.3
Male (untreated)	8	31 ± 2.1	1.5 ± 0.4	NA

Table 32: Summary of kinetics with pregnenolone in bovine liver cytosol.

Each assay was carried out in duplicate. V_{max}, K_m and K_i values ± standard error for the liver samples is shown.

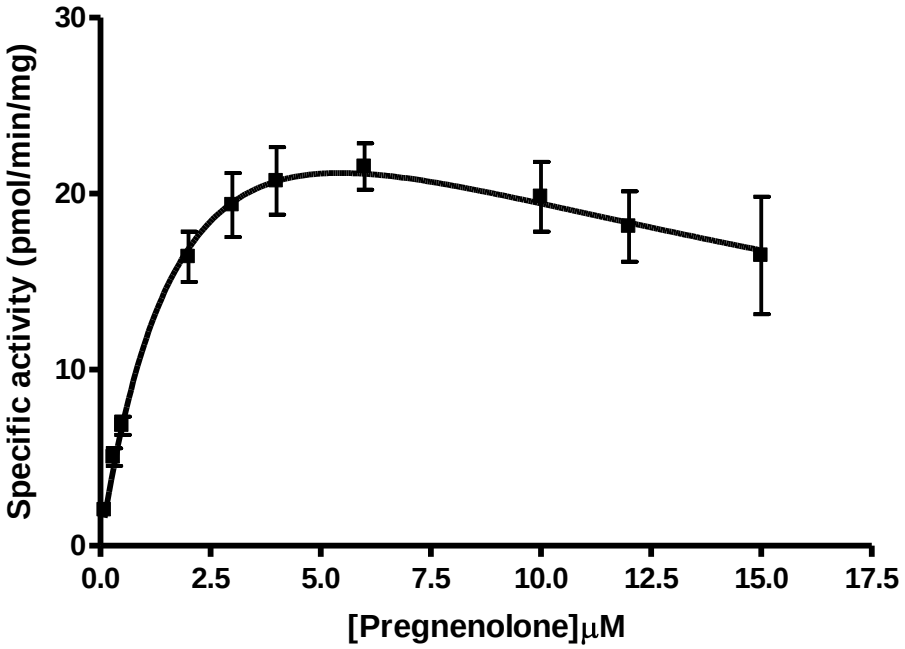


Figure 38: Enzyme kinetics of pregnenolone in untreated female bovine liver cytosol.

Rate of formation of pregnenolone sulfate as a function of pregnenolone concentration in female (untreated) bovine liver cytosol. Data points are ± standard error. n=8

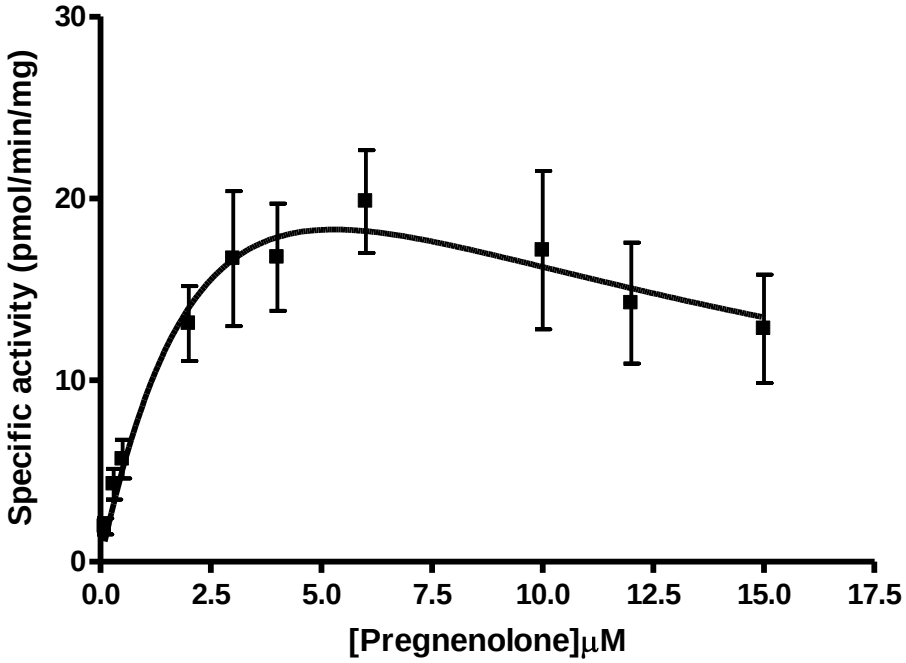


Figure 39: Enzyme kinetics of pregnenolone in treated female bovine liver cytosol

Rate of formation of pregnenolone sulfate as a function of pregnenolone concentration in female (treated) bovine liver cytosol. Data points are ± standard error. n=4

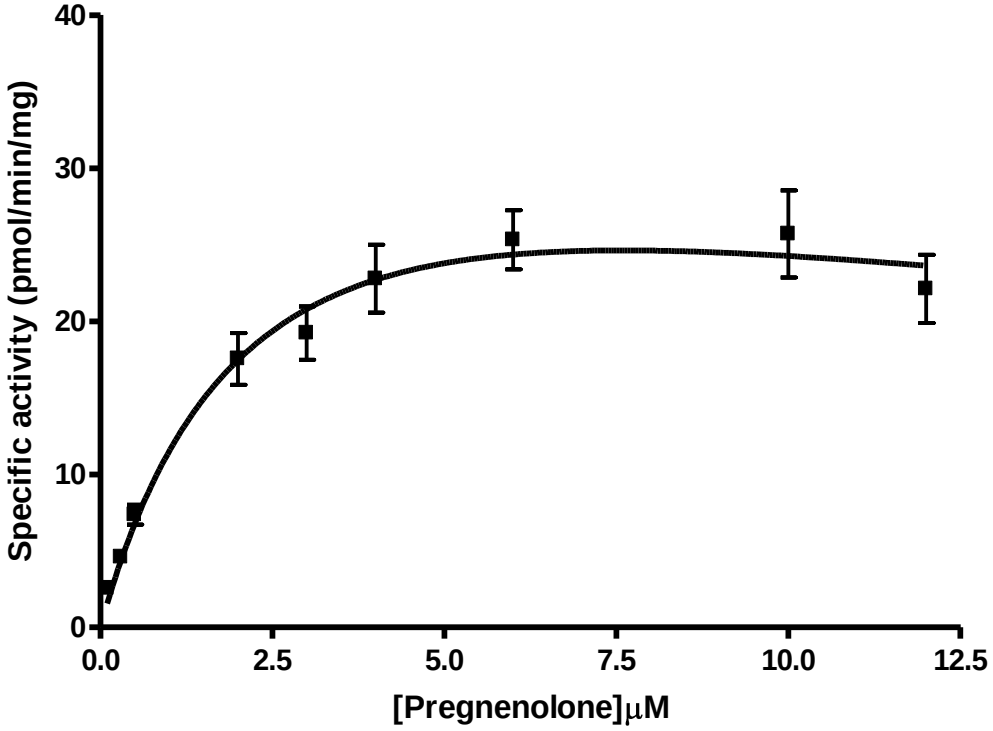


Figure 40: Enzyme kinetics of pregnenolone in male bovine liver cytosol

Rate of formation of pregnenolone sulfate as a function of pregnenolone concentration in male bovine liver cytosol. Data points are ± standard error, n=8

4.5 Detection of expression of SULT1B1 in bovine liver using anti human SULT1B1 peptide antibody.

SULT1B1, the major isoform of the SULT1B family and the second most abundant SULT1 enzyme present in human liver is known to have broad substrate specificity towards a range of phenolic compounds such as 4-nitrophenol, 2-aminophenol and 1-naphthol. It has huge overlapping substrate specificities with SULT1A1, the major drug metabolising enzyme present in human liver (Riches et al., 2009). This makes finding an exclusive substrate to assess the activity of SULT1B1 in liver or other drug metabolising tissues extremely difficult. 4-nitrophenol was used as a substrate of choice to probe SULT1B1 activity in bovine livers. Using anti-human SULT1A3 and anti-minipig SULT1A1 antibody raised against purified recombinant human SULT1A3 and minipig SULT1A1 respectively, expression of SULT1A1 was not detected in any of the bovine livers. Lack of expression of SULT1A1 in bovine liver suggested that SULT1B1 could possibly be the major enzyme involved in 4-nitrophenol metabolism although there might be other SULT isoforms that contribute towards 4-nitrophenol metabolism in the bovine liver such as members of the SULT1C family (Tabrett and Coughtrie, 2003).

SDS-PAGE gel electrophoresis was performed in a similar manner to that described earlier in this chapter. Immunoblot analysis was carried out using a multiple antigenic peptide (MAP) antibody raised against a specific unique sequence in human SULT1B1 (Riches et al., 2009). The antibody was cleared against bovine serum proteins to reduce nonspecific binding. SULT1B1 was detected in both male as well as female livers with considerable amount of variation in each set of livers (figure 41). A difference as high as 30-fold was

observed between the untreated female livers and a 7 fold difference between bSULT1B1 expressions in male bovine liver was seen. Fold difference was calculated by subtracting the lowest value from the highest one and dividing it by the number of samples. On calculation of average band densities for male and female bovine livers, no significant sex difference was noted (figure 42). This was proved statistically using a 2-tailed t-test assuming unequal variance (table 33). Comparison of untreated and treated female bovine livers revealed a 32 fold difference between the untreated female livers and a 1.5 fold difference between the treated. Upon calculation of average band densities, it was found that average expression of SULT1B1 in the treated livers was 3 times lower than the average expression in the untreated livers (table 33). However, due to the high amount of variation seen in the two sets of female livers it would be inappropriate to link this finding to differential SULT1B1 expression between the untreated and treated livers especially in such a small sample size.

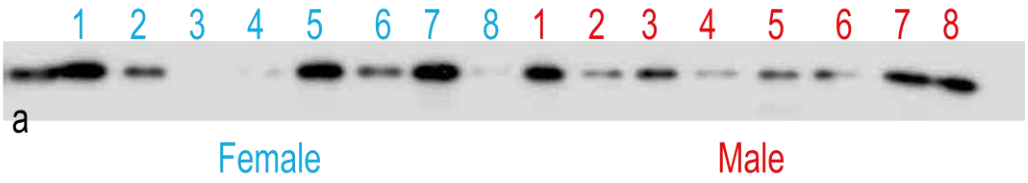


Figure 41: SULT1B1 expression in bovine livers.

Bovine SULT1B1 expression in 8 untreated female and 8 untreated male livers. 30µg of liver cytosol was loaded for every liver sample. ***a*** *contains 0.1 µg of recombinant bovine SULT1B1.*

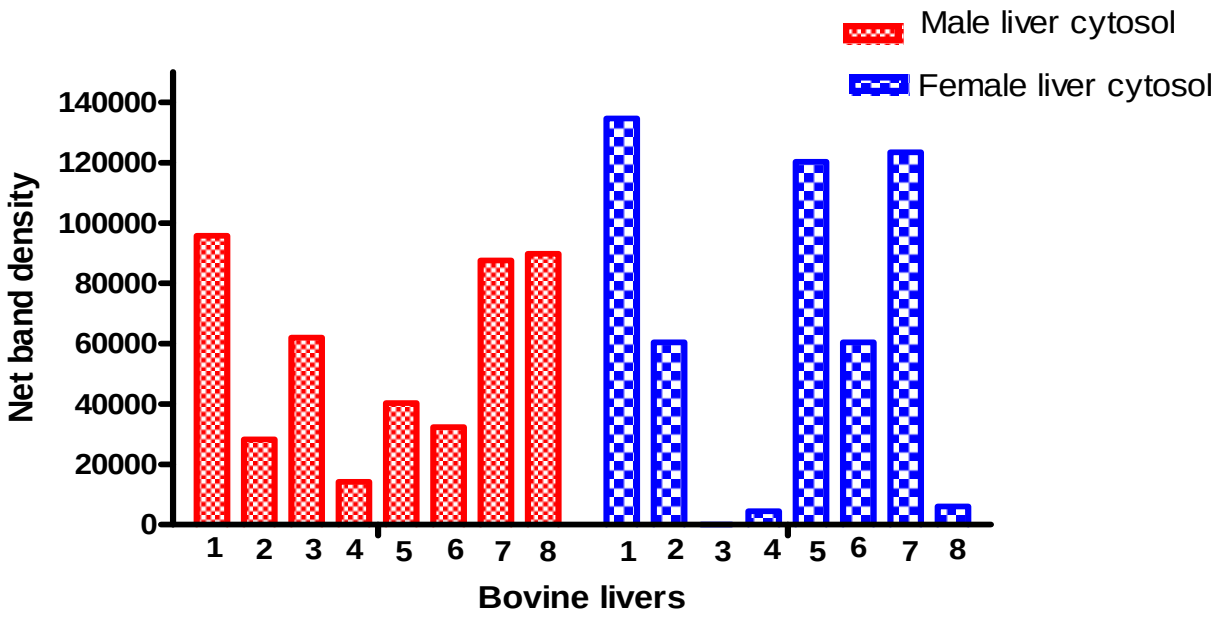


Figure 42: Net band density values as an indicator of SULT1B1 expression in male and female bovine liver.

Graph above shows the net band density values (bovine SULT1B1 expression) for the 8 untreated male and female bovine liver samples.

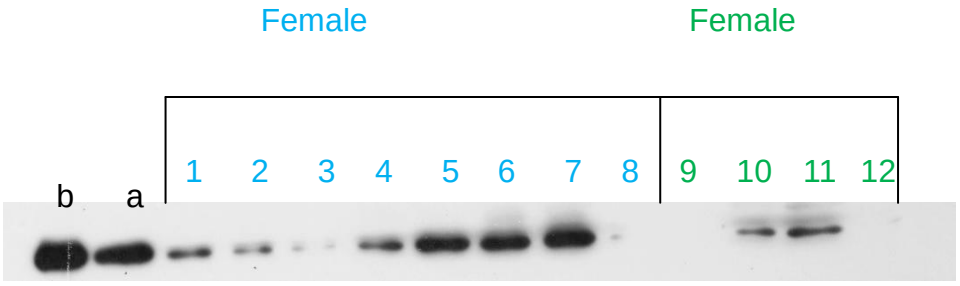


Figure 43: Bovine SULT1B1 expression in 8 untreated and 4 treated female livers.

30µg of liver cytosol was loaded for every liver sample. ***a, b*** *contains 0.2 µg and 0.5µg of recombinant bovine SULT1B1 respectively.*

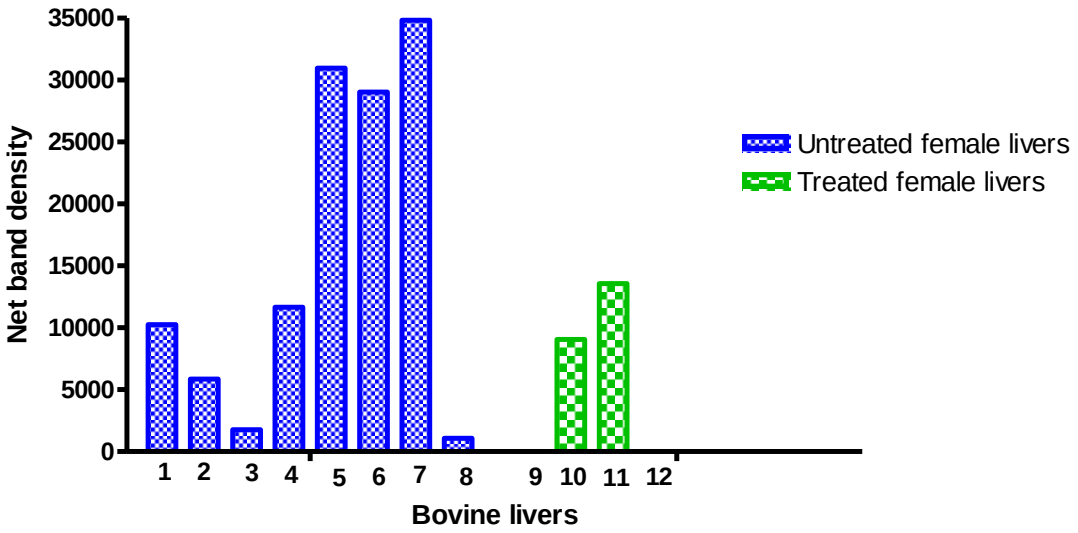


Figure 44: Net band density values as an indicator of SULT1B1 expression in untreated and treated female bovine liver.

Net band density values for bovine SULT1B1 expression in 8 untreated female (1-8) and 4 treated female (9-12) bovine liver samples.

Liver cytosol	No of samples (n)	Average	Standard deviation	P value	Result
Female (UT)	8	113770	78910	0.09	Null hypothesis accepted
Female (T)	4	42170	51980		
Male (UT)	8	56280	31800	0.75	Null hypothesis accepted
Female (UT)	8	63700	56950		

Table 33: Expression levels (average band densities) of bSULT1B1 in male and female bovine liver samples.

UT= Untreated and T= Treated. Null hypothesis (H_o) = There is no difference in expression levels of bSULT1B1 between male and female or between female (UT) and female (T). A two tailed t-test assuming unequal variance was carried out. Null hypothesis was rejected if $P < 0.05$

4.6 Analysis of bovine SULT1B1 activity towards 4-nitrophenol in bovine liver samples

Using conditions optimized in table 30, kinetic analysis was performed on male and female bovine livers using 4-nitrophenol as the substrate. Once again, the female bovine livers demonstrated partial substrate inhibition (figure 45 and 46) whereas the male livers followed Michaelis-Menten kinetics (figure 47). No significant difference was seen in the V_{max} values between male and female livers. Considerable amount of variation was seen in activity towards 4-nitrophenol between female bovine livers as compared to their male counterpart. Given below is a table listing the V_{max}, K_m and K_i values for 4-nitrophenol in bovine liver tissue.

Liver	No of samples (n)	Vmax (pmol/min/mg)	K_m (µM)	K_i (µM)
Female (untreated)	8	3071 ± 3601	22.6 ± 31.4	4.8 ± 7.1
Female (treated)	4	1411 ± 903	6 ± 6	14 ± 15.5
Male (untreated)	8	3621 ± 236.7	12.9 ± 1.6	-

Table 34: Summary of kinetics with 4-nitrophenol in bovine liver cytosol.

Each assay was carried out in duplicate. V_{max}, K_m and K_i values ± standard error for the liver samples is shown.

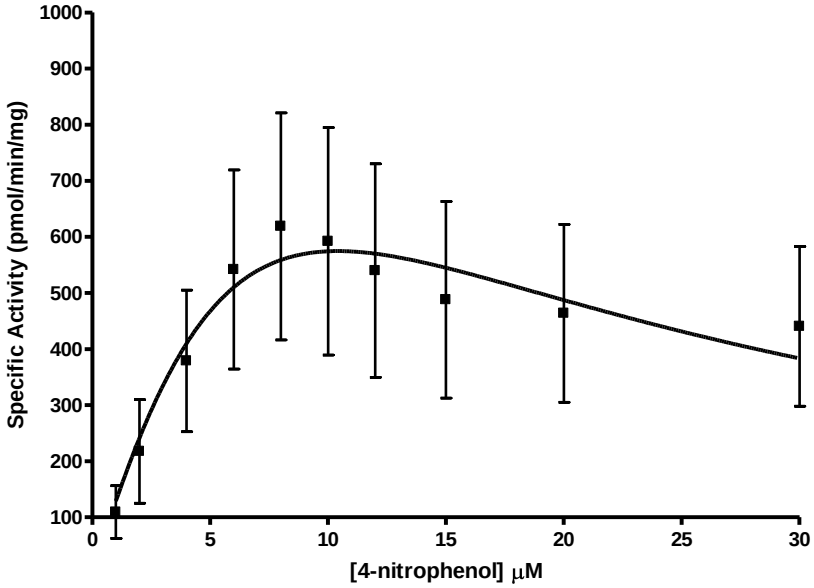


Figure 45: Kinetics of 4-nitrophenol in female (untreated) bovine liver cytosol

Rate of formation of 4-nitrophenol sulfate as a function of 4-nitrophenol concentration in female (untreated) bovine liver cytosol. Data points are ± standard error. n =8

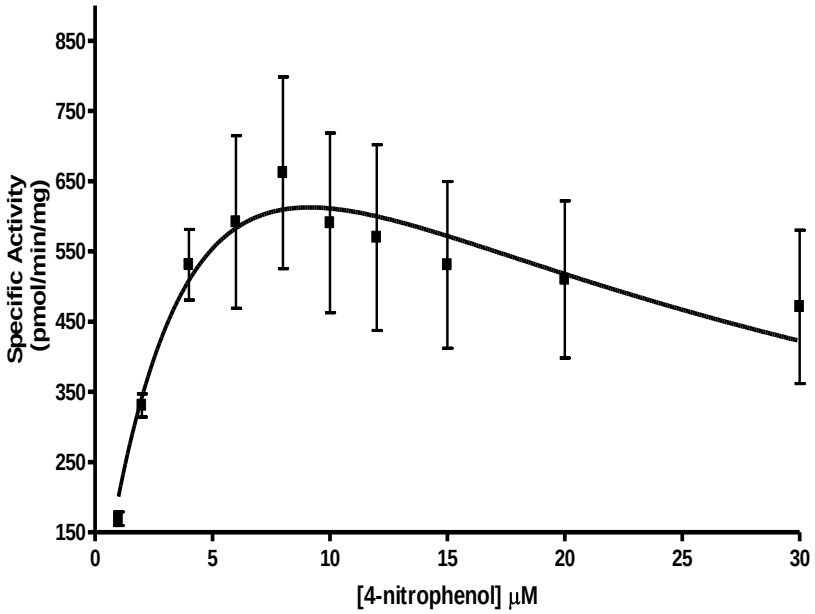


Figure 46: Kinetics of 4-nitrophenol in female (treated) bovine liver cytosol

Rate of formation of 4-nitrophenol sulfate as a function of 4-nitrophenol concentration in female (treated) bovine liver cytosol. Data points are ± standard error, n=4.

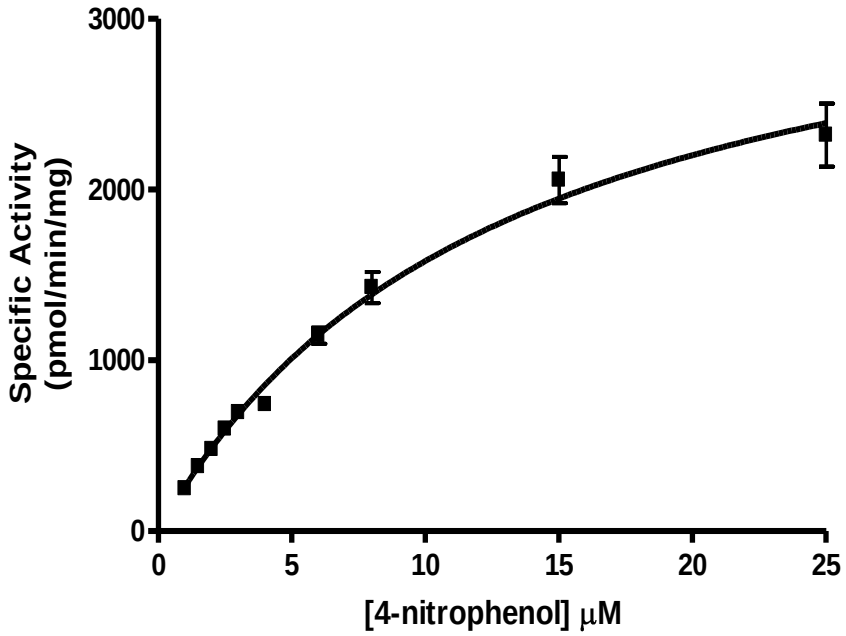


Figure 47: Kinetics of 4-nitrophenol in male bovine liver cytosol

Rate of formation of 4-nitrophenol sulfate as a function of 4-nitrophenol concentration in male bovine liver cytosol. Data points are ± standard error, n=8.

4.7 Detection of expression of SULT1E1 in bovine liver using anti human SULT1E1 antibody.

In humans, estrogen sulfotransferase (SULT1E1), unlike SULT1B1 or SULT1A1 is highly specific and almost exclusively metabolises its substrate 17β-estradiol in the nanomolar concentration range. Immunoblot analysis was carried out using anti-human SULT1E1 antibody raised against purified recombinant SULT1E1. The anti-human SULT1E1 used cross reacts with bSULT1B1 and bSULT1A1. Although all the 3 bands are distinguishable from each other on a western blot, desitometric scanning using QuantiScan becomes difficult. Since expression levels vary significantly between samples, it becomes difficult to load equal amounts of protein on the gel without compromising with the clarity of the western blot. For example loading 30µg of F10 instead of the 3µg that was actually loaded creates a strong band that smears into the adjacent lane thus making it difficult to see bSULT1E1 expression clearly. If different amount of protein is loaded per sample then making a fair comparison with desitometric scanning would not be possible. Hence it was decided to not analyse the bSULT1E1 blots using QuantiScan.

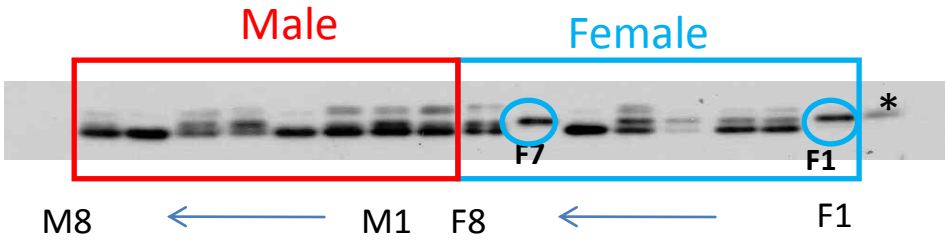


Figure 48: Bovine SULT1E1 expression in 8 untreated female and male livers.

Male livers M1-M8 and female livers are F1-F8. 30µg of liver cytosol was loaded for every liver sample except F1 (10µg) and F7 (5µg). ***** *contains 0.1µg of recombinant bovine SULT1E1.*

Expression of bSULT1E1 was clearly detected in some of the untreated female bovine livers such as F1 and F7 (figure 48). Expression was also detected in livers F2, F3, F4 and F5 but to a much lesser extent than F1 and F7. F1 and F7 were loaded at 10 and 5µg respectively compared to the rest that were loaded to a final concentration of 30 µg. Slight expression of bSULT1E1 was probably seen in some of the male livers such as M1, M2, M5 and M6. However, presence of multiple bands with higher intensity than the band of interest makes it difficult to have confidence in the expression of bSULT1E1 in the male liver.

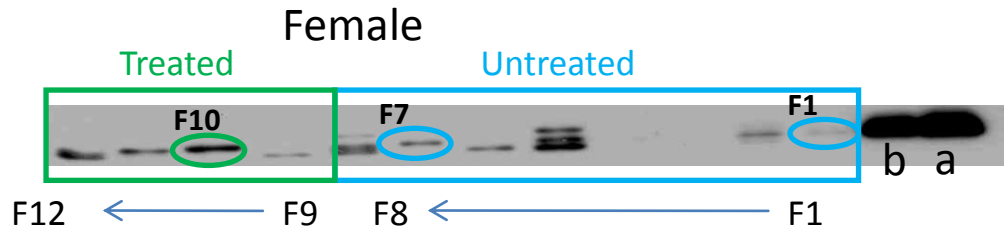


Figure 49: Bovine SULT1E1 expression in 8 untreated female and 4 treated female livers.

30µg of liver cytosol was loaded for every liver sample except F1 (10µg), F7 (5µg) and F10 (3µg). a and b contains 0.5, 0.3 µg of recombinant bovine SULT1E1 respectively.

Within the treated female bovine livers, expression of bSULT1E1 was strongly detected in liver F10 (figure 49). Only 3µg of F10 was loaded to give rise to a strong band seen in figure 4.16. Slight expression was also detected in liver F11. Considerable amount of variation was seen in bSULT1E1 expression in the female liver, with expression only in livers F1, F2, F5 and F7.

4.8 Analysis of bovine SULT1E1 activity towards 17β-estradiol in bovine liver samples

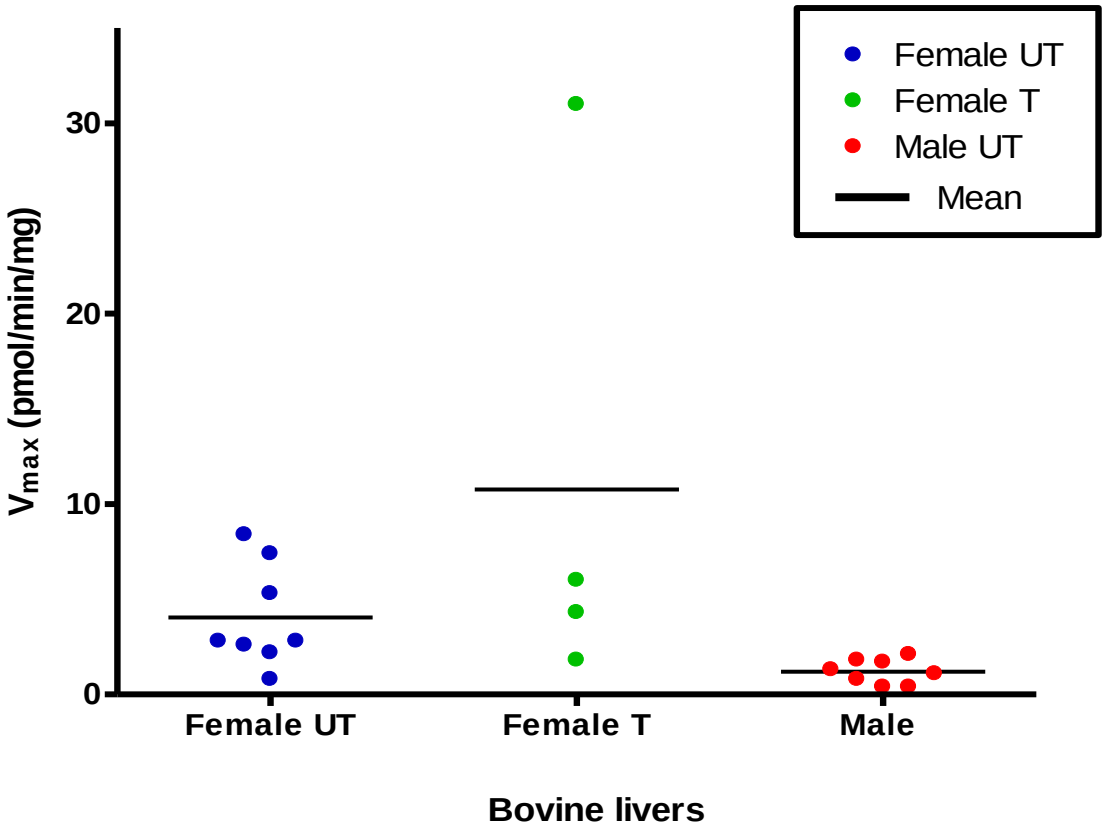
Both male and female livers followed Michaelis-Menten kinetics for enzyme activity towards 17β-estradiol. Activity in the male liver was lower than the activity in female livers. A two-tailed t-test assuming unequal variance was

carried out (see figure 50) which supported this initial observation. Activity in female livers treated with the exogenous progestin had thrice as much activity towards 17β-estradiol as compared to the untreated bovine livers; however from table 36 it appears that liver F10 was alone responsible for the high level of activity seen with 17β-estradiol in treated female livers (see region highlighted in yellow in table 35). A two tailed t-test assuming unequal variance showed that the difference in activities of untreated and treated female livers was statistically insignificant. Variability in female livers ranged from 0.8-31 pmol/min/mg whereas those in male livers ranged from 0.8-1.8 pmol/min/mg (table 35). Individual analysis of activity led to the observation that only certain livers such as F10 > F1 and F7 had a high level of activity.

Liver	V_{max} (pmol/min/mg)	K_m (μM)
F1	8.4 ± 0.5	0.03 ± 0.01
F2	5.3 ± 2.8	0.8 ± 0.7
F3	2.8 ± 0.2	0.05 ± 0.02
F4	2.6 ± 0.3	0.1 ± 0.04
F5	2.2 ± 0.13	0.03 ± 0.01
F6	2.8 ± 0.14	0.1 ± 0.02
F7	7.4 ± 0.4	0.03 ± 0.006
F8	0.8 ± 0.1	0.02 ± 0.02
F9	4.3 ± 2.4	0.6 ± 0.6
F10	31 ± 1.4	0.14 ± 0.02
F11	6 ± 1	0.4 ± 0.1
F12	1.8 ± 0.6	0.2 ± 0.2
M1	0.8 ± 0.2	0.2 ± 0.1
M2	2.1 ± 0.2	0.1 ± 0.02
M3	0.4 ± 0.1	0.07 ± 0.04
M4	0.4 ± 0.1	0.05 ± 0.03
M5	1.3 ± 0.12	0.05 ± 0.02
M6	1.1 ± 0.14	0.05 ± 0.02
M7	1.8 ± 0.6	0.4 ± 0.2
M8	1.7 ± 0.05	0.1 ± 0.007
Female (untreated) n=8	4 ± 0.5	0.04 ± 0.02
Female (treated) n=4	11 ± 4.7	0.2 ± 0.2
Male (untreated) n=8	1.2 ± 0.2	0.08 ± 0.05

Table 35: Kinetics with 17β-estradiol as the substrate for each of the 20 liver samples analysed individually.

F1-F8 are the 8 untreated bovine liver samples. F9-F12 are 4 female bovine liver samples treated with an exogenous progestin. M1-M8 are the 8 male liver samples. All female (untreated), female (treated) and male bovine livers were also analysed together. Each assay was carried out in duplicate. V_{max}, K_m and K_i values ± standard error for each of the liver samples is shown.



Liver cytosol	No of samples (n)	Average V_{max} (pmol/min/mg)	STDEV	T-test unequal variance	Result
Female (UT)	8	4.03	2.70	0.40	Null hypothesis accepted
Female (T)	4	10.78	13.59		
Male (UT)	8	1.20	0.64	0.02	Null hypothesis rejected
Female (UT)	8	4.03	2.70		

Figure 50: Activity towards 17β-estradiol in male and female bovine livers.

Scatter plot showing distribution of V_{max} for each of the livers. UT= Untreated and T= Treated. Null hypothesis (H_o) = There is no difference in the activity towards 17β-estradiol between male and female livers. A two tailed t-test assuming unequal variance was carried out. Null hypothesis was rejected if P < 0.05.

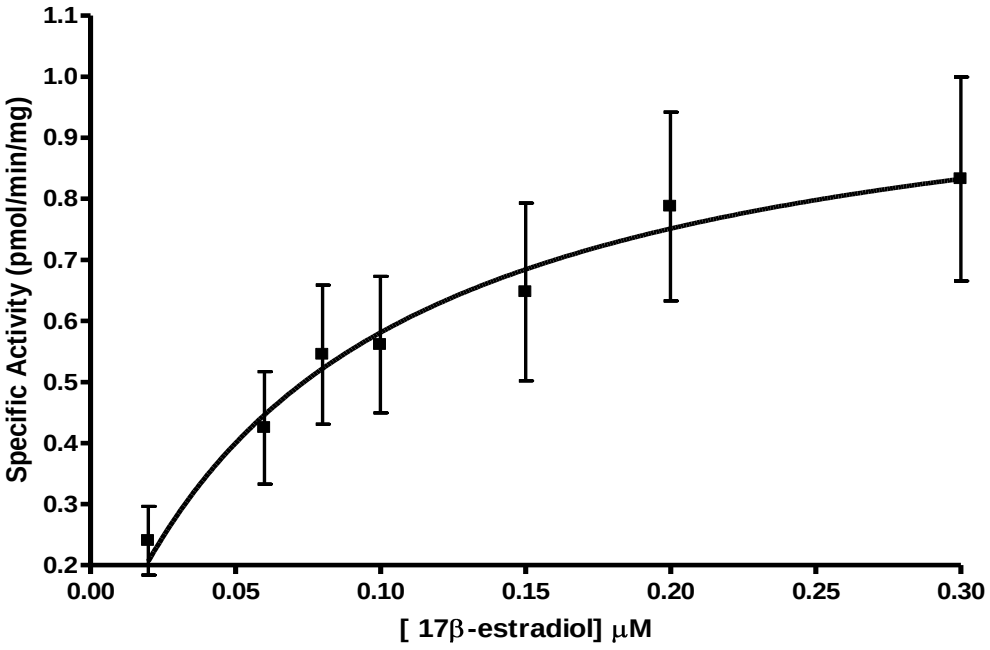


Figure 51: Kinetics of 17β-estradiol in male bovine liver cytosol

Rate of formation of 17β-estradiol sulfate as a function of 17β-estradiol concentration in male bovine liver cytosol. Data points are ± standard error, n=8.

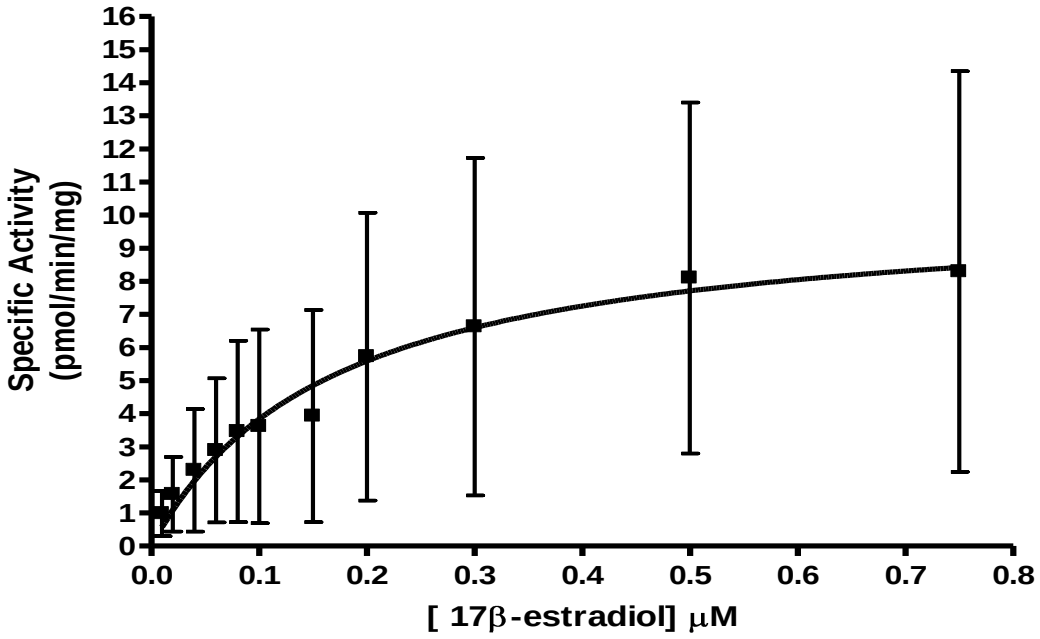


Figure 52: Kinetics of 17β-estradiol in female (treated) bovine liver cytosol

Rate of formation of 17β-estradiol sulfate as a function of 17β-estradiol concentration in female (treated) bovine liver cytosol. Data points are ± standard error, n=4.

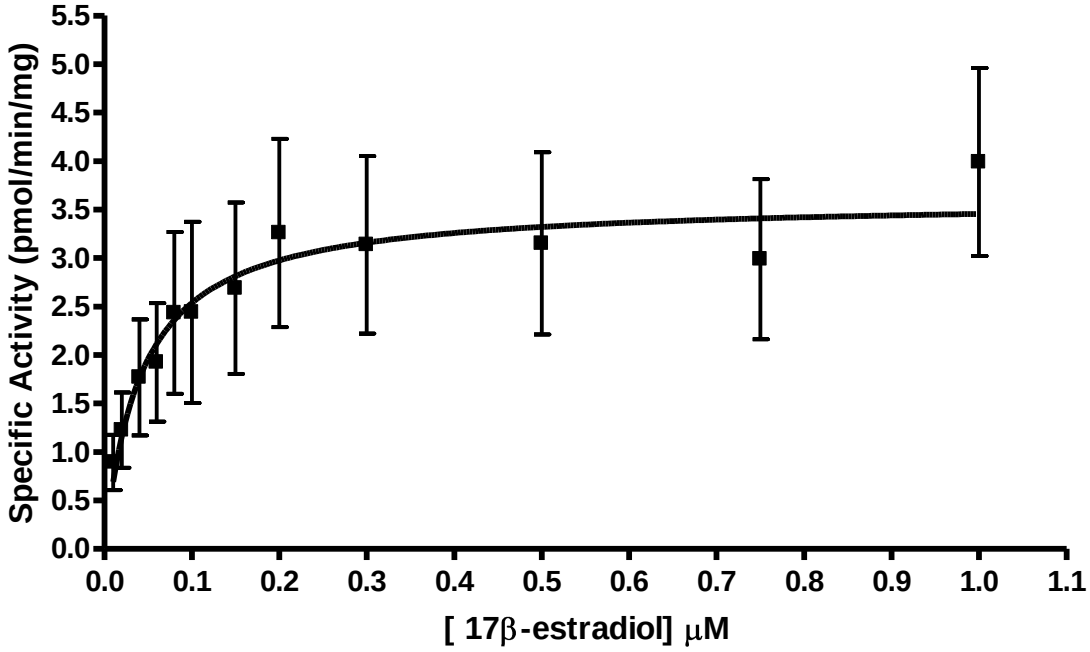


Figure 53: Kinetics of 17β-estradiol in female (untreated) bovine liver cytosol

Rate of formation of 17β-estradiol sulfate as a function of 17β-estradiol concentration in female (untreated) bovine liver cytosol. Data points are ± standard error, n=8.

4.9 Substrate specificity profiling with bovine liver

Comparison of sulfation between the 3 different categories of livers might provide useful insights in SULTs that might be involved in sulfation in male and female bovine livers. A substrate screen using a battery of xenobiotic compounds (mostly phenolic compounds given their compatibility with [^{35}S] assay) was carried out on male and female bovine liver cytosol. The compounds chosen to be screened were selected on the basis of activity seen with the compounds in human liver cytosol. The same assay conditions were applied to both male and female livers. Activity assays were performed using the same optimised conditions that were applied to kinetic reactions (table 30). Male bovine livers were better at sulfation compared to the female livers (figure 54 and 55). Male

bovine livers had twice as much activity towards most of the substrates compared to the female livers. Substrates such as paracetamol, dobutamine and apomorphine were not metabolised in both sets of livers. No significant difference was observed in bovine female livers treated with an exogenous progestin as compared to the untreated ones (figure 55 and 56). Rate of sulfation of 4-nitrophenol and 2-aminophenol in the male bovine liver was almost similar However, in the female liver, both treated and untreated rate of 2-aminophenol sulfation was twice as much as that of 4-nitrophenol sulfation.

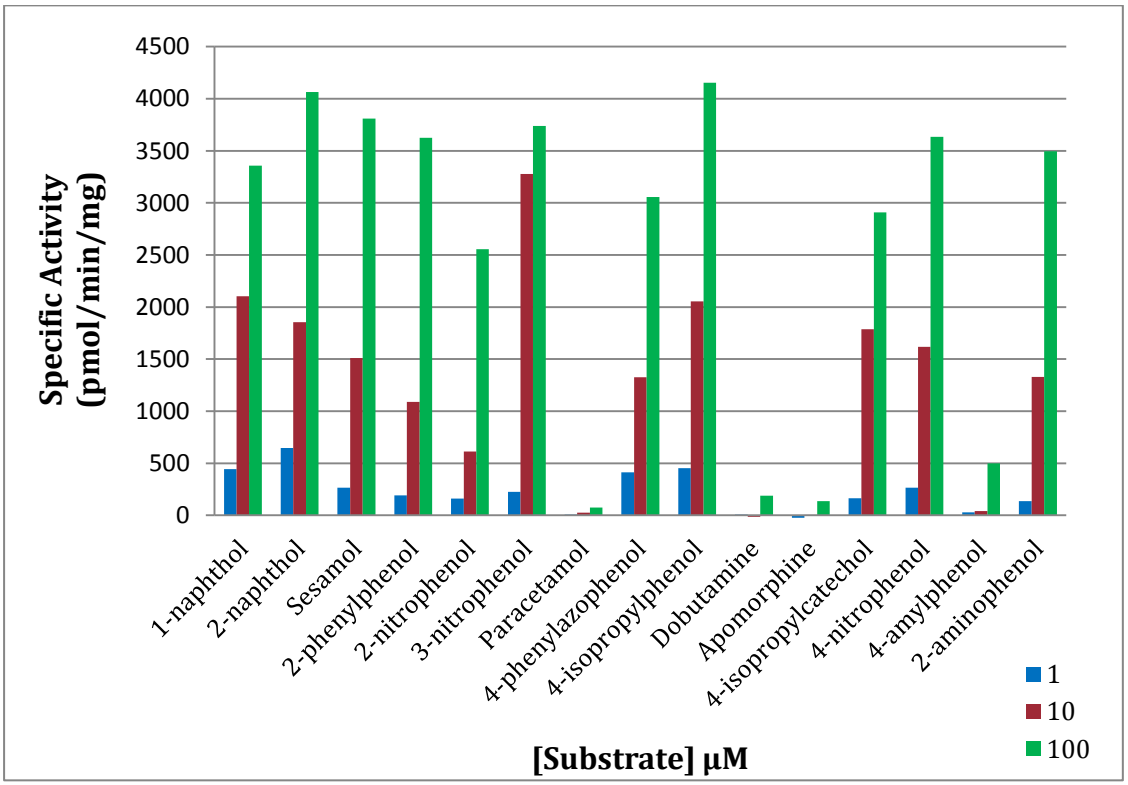


Figure 54: Substrate specificity profiling in bovine male liver cytosol using phenolic substrates

A battery of phenolic substrates was screened for activity in 30µg of bovine male liver cytosol using the [^{35}S] PAPS assay. The substrates were used at 1, 10 and 100µM concentrations.

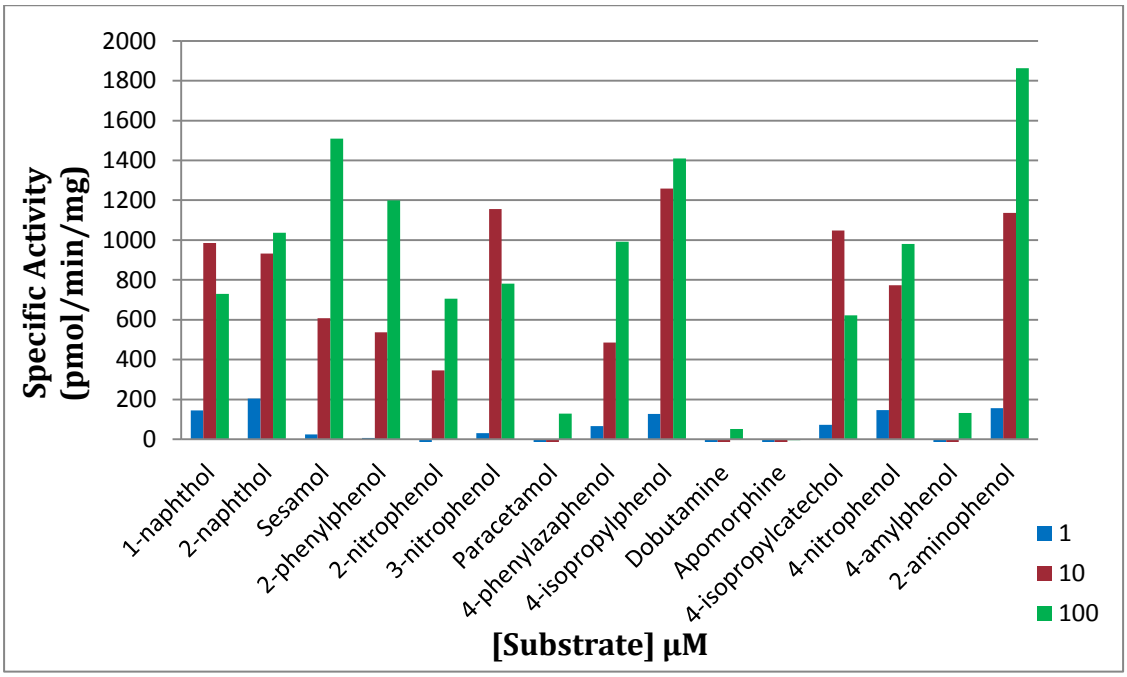


Figure 55: Substrate specificity profiling in bovine female (untreated) liver cytosol using phenolic substrates

A battery of phenolic substrates was screened for activity in 30µg of bovine female liver (untreated) cytosol using the [^{35}S] PAPS assay. The substrates were used at 1, 10 and 100µM concentrations.

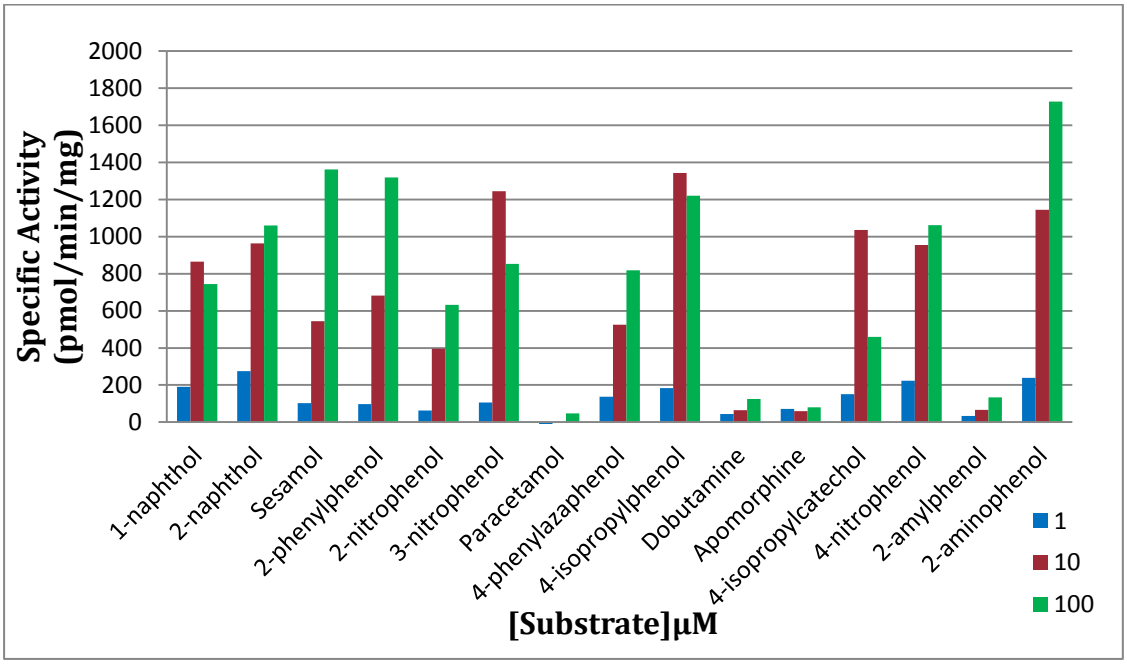


Figure 56: Substrate specificity profiling in bovine female (treated) liver cytosol using phenolic substrates

A battery of phenolic substrates was screened for activity in 30µg of bovine female liver (treated) cytosol using the [^{35}S] PAPS assay. The substrates were used at 1, 10 and 100µM concentrations.

4.10 Discussion

The ability to reliably predict the *in vivo* fate of drugs in livestock would be of considerable value to the pharmaceutical, livestock industry as well as the regulatory bodies. Since sulfation is considered to be one of the major drug and xenobiotic clearance pathway in humans and other species it is important to understand drug metabolism brought about by sulfotransferases in the liver which is the prime organ involved in drug metabolism. In this chapter, expression and activity of 4 major drug metabolising isoforms namely SULT1B1, SULT1E1, SULT2A1 and SULT1A1 have been investigated. Substantial variation in expression was noted among bovine livers for all the isoforms studied. Variation in expression was especially high for female bovine livers compared to the male. A significant sex difference was seen in bovine SULT2A1 expression in liver but no difference was noted in activity towards pregnenolone.

4.10.1 Presence of SULT1A1 and SULT1B1 in bovine liver

Although metabolised by SULT1B1, 2-aminophenol is a more specific substrate for human SULT1A1. It has a higher V_{max} and a lower K_m for SULT1A1(Riches et al., 2007)**.** 2-aminophenol has a much higher intrinsic clearance value with SULT1A1 compared to SULT1B1 suggesting that in cytosol where many other SULTs are present, 2-aminophenol sulfation measured would be predominantly due to SULT1A1 activity. It was also shown that in human liver cytosol where both SULT1B1 and SULT1A1 are present SULT1B1 had a greater influence in sulfating 4-nitrophenol than compared to 2-aminophenol (Riches et al., 2007). Results from the substrate screen here showed that 2-aminophenol was metabolised twice as much as 4-nitrophenol in the female liver. In the bovine male liver, no significant difference was observed in the sulfation of the two

substrates. Moreover, kinetic studies carried out in the previous chapter on recombinant bovine SULT1A1 and SULT1B1 with 4-nitrophenol as the substrate showed that bovine SULT1A1 underwent partial substrate inhibition whereas SULT1B1 followed Michaelis-Menten kinetics. Male bovine liver cytosol also demonstrated Michaelis-Menten kinetics with similar V_{max} values and kinetics of 4-nitrophenol with female bovine liver cytosol demonstrated partial substrate inhibition. Activity data analysis indicates that probably SULT1A1 is the major drug metabolising enzyme present in the female bovine liver whereas SULT1B1 might be the major one in the male bovine livers. Immunoblot analysis carried out with anti-human SULT1A3 and anti-minipig SULT1A1 antibody have not been able to detect the presence of the SULT1A1 isoform in the male and female liver. Bovine SULT1B1 was detected in both the sexes using anti human SULT1B1 peptide antibody. There is a lot of sequence similarity between the human, minipig and bovine SULT proteins. Although the antibodies used cross react with bovine SULTs, they might not be as sensitive to SULT expression in cattle as compared to their own SULT isoforms. In such a case it is difficult to correlate expression with activity. Development of bovine SULT isoform specific antibodies might provide deeper insights into expression of SULT1A1 and SULT1B1 in the bovine liver.

4.10.2 SULT1E1 in bovine liver

17β-estradiol is known to be a good probe substrate for SULT1E1 since it is highly specific for 17β-estradiol in the nanomolar concentration range found in physiological conditions (Kester et al., 1999b; Song and Melner, 2000). Other SULT isoforms such as SULT1A1 and SULT1B1 also metabolise 17β-estradiol but do so in the micromolar range (Kester et al., 1999b) . SULT1E1 is expressed in the

endometrium in humans where it is known to regulate estrogen action during the menstrual cycle, possibly under the influence of progesterone (Rubin et al., 1999). Hormones and other factors such as environmental pollutants like polychlorinated biphenyls which inhibit SULT1E1 activity (Kester MHA, 2000) and bring about endocrine disrupting effects (Safe, 1994) might be responsible for the variation seen in its expression in female liver. Clear sex related differences were noted in the expression and activity of SULT1E1 in bovine liver. However, like other SULTs it was not possible to analyse the western blots for band density using QuantiScan. Although highly variable, female liver had a higher overall expression of SULT1E1 compared to the males. This is opposite to what has been observed in rat and mice where SULT1E1 expression is also sexually dimorphic but more prominent in sexually mature and competent males (Demyan WF, 1992; Song W-C, 1997). In humans, SULT1E1 expression is the greatest in fetal development and decreases with age. The expression was also found to be highest in foetuses of the male gender (Duanmu et al., 2006) . It is quite possible that high levels of SULT1E1 in immature males are responsible for the inactivation of estrogens, thereby increasing androgen intensity during gonadal organogenesis and a lack of SULT1E1 expression in the mature bovine male liver is matter of age (Duanmu et al., 2006)

4.10.3 SULT2A1 in bovine liver

Expression of SULT2A1 was detected in both male as well as female bovine liver using anti human SULT2A1 antibody. However, activity towards DHEA, its probe substrate was not detected in the bovine liver. Structural studies on human SULT2A1 proposed that Met137 was responsible for the regulation of binding orientation of DHEA (Lu-Yi Lu, 2007). In bovine SULT2A1, Met137 is replaced by

alanine (chapter 3). It is quite possible that the substitution might affect DHEA binding in cattle SULT2A1. SULT2A1 also turns over pregnenolone (Fuda et al., 2002). This was used to assess SULT2A1 activity in the bovine liver. Expression of SULT2A1 in bovine male liver was twice as much as the female liver. However, no major difference in activity was noted. Sex related differences in SULT2A1 expression have also been observed in rat liver where females had higher expression levels than males (Rajkowski et al., 1997). SULT2A1 expression in rats is also age dependent being very high during fetal development where the liver is more androgen insensitive The expression levels drop in mature rats as the liver becomes more androgen sensitive (Roy, 1992a). The kinetics of sulfation of pregnenolone found in the bovine male liver was distinct from that in the female liver. Strong substrate inhibition was seen in the female liver but not in the male liver indicating that different enzymes or set of enzymes is involved in pregnenolone metabolism in bovine liver. Pregnenolone in the human liver is also metabolised by SULT2B1 (Fuda et al., 2002). The gene encoding bovine SULT2B1 is present in cattle liver (Zimin et al., 2009) but the presence of bovine SULT2B1 in the bovine liver has not yet been established, however a possibility of its existence remains.

4.10.4 Conclusion

Although human SULT antibodies used in this study cross reacted with the bovine SULT proteins, they do not bind as specifically and strongly as they would to human SULT proteins. Hence expression and activity of bovine SULTs should be compared with caution. In some cases where expression was not seen activity was still detected. For example expression of bSULT2A1 was not detected in F1 and F3 however activity was still found. Except for 17β-estradiol which is highly

specific for SULT1E1 in the nanomolar range, most of the substrates used in this study were not exclusively metabolised by their respective isoforms. In humans and rat pregnenolone is extensively metabolised by SULT2B1 (Fuda et al., 2002) and 4-nitrophenol is turned over by several different members of the SULT1 family (Tabrett and Coughtrie, 2003) and hence the two are not ideal substrates for studying SULT2A1 and SULT1B1 metabolism in bovine liver. Pregnenolone was used because DHEA, the human probe substrate for SULT2A1, is not metabolised by the liver or recombinant bovine SULT2A1. SULT1B1 metabolises the majority of the substrates turned over by SULT1A1. To date no known probe substrate that is exclusively turned over by SULT1B1 has been discovered. In the future to study drug metabolism brought about in the liver or any other drug metabolising tissue by SULTs it would be advantageous to have anti bovine SULT antibodies and probe substrates that would be exclusively turned over by their respective SULT isoforms (Roy, 1992a).

5 Conjugative metabolism of estradiol *in vitro*

5.1 Introduction

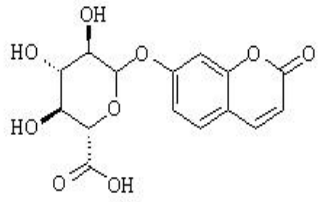
Estrogens influence the growth, differentiation and functioning of target tissues such as the breast, ovaries, testis and prostate. They are produced in both male and female reproductive organs from where they rapidly diffuse in and out of the cells. They are retained in target cells by binding with high affinity to intracellular estrogen receptors (ER). Binding of estrogen causes the ER to undergo a conformational change which then binds to the chromatin to modulate transcription of target genes (Raftogianis et al., 2000). 17β-estradiol is the biologically active form of estrogen found endogenously. It is also a very potent stimulator of the ER. 17β-estradiol has been used clinically in the treatment of hormonal disorders in women especially in hormone replacement therapy (Raftogianis et al., 2000). In livestock it is used for growth promotion, improving feed efficiency and also in estrus synchronisation. 17β-estradiol is usually administered as a pro drug; estradiol benzoate. Studies have shown that estradiol benzoate is rapidly converted to 17β-estradiol at the site of injection or in blood. In ruminants, 17β-estradiol is converted to estrone and then to 17α-estradiol in a series of oxidation reduction reactions. The 17α-estradiol is then subsequently conjugated (Dunn, 1977). Subcutaneous implants at the rear side of the ear are the preferred route of application of steroid hormones since dose delivered over a period of time can be controlled by altering the implant matrix properties. Desired anabolic results can be achieved and at the same time accumulation of high amounts of unwanted residues in edible tissues can be avoided by using this route of administration. Estrogen is a requirement to elicit growth response hence all steroid compounds such as testosterone and androsterone used commercially are usually given with an estrogenic compound (Maume et al., 2001). Due to the carcinogenic potential of these steroid hormones including

estradiol, their use as growth promoting agents is banned in the EU. However, their use is still legal in some nations like the USA, Australia and New Zealand. However, this is subject to strict regulation on the amount of these residues present in edible tissues such that residues of estradiol are permitted only in the parts per trillion range (Wayne, 1986) . A considerable amount of information is available on estrogen metabolites in reproductive tissues but very little on edible tissues in animals (Mellin and Erb, 1965; Velle, 1963). In order to ensure human food safety of animal derived food products, it is necessary for the pharmaceutical industry to obtain information on the metabolism of estradiol. In order to do so it must develop *in vitro* model systems that can reliably predict *in vivo* metabolism in target species. The aims of the following series of experiments were to evaluate microsomes, cytosol and cryopreserved bovine hepatocytes as *in vitro* models for studying the phase 2 metabolism of 17β/α-estradiol and compare results to existing data available on *in vivo* metabolism of estradiol.

5.2 Strategy adopted

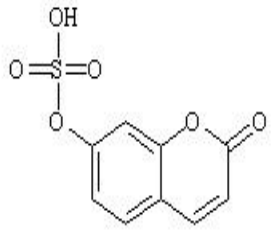
Conjugative metabolism of 17β-estradiol and 17α-estradiol brought about by glucuronidation and sulfation were studied in the above mentioned model systems using Ultra Performance Liquid Chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Chromatographic conditions were optimized for internal standards (figure 57) and standard metabolites used (Figure 58). 17β/α-estradiol was incubated with microsome, cytosol and hepatocyte preparations at 37°C with the appropriate reagents and the samples were subsequently run on a UPLC-MS/MS platform along with the standard metabolites and internal standards. Identification of metabolites generated in the test samples was done based on similar retention times with the available

standards. After time and protein concentration optimization, kinetics was performed wherever possible, on the detected metabolites.



7-hydroxycoumarin-glucuronide

(7OHCG) MW; 338.27



7- hydroxycoumarin-sulfate

(7OHCS) MW; 242.21

Figure 57: Chemical structures and molecular weight of internal standards used

5.3 Use of an Internal Standard

An internal standard is a compound that is added in equal quantities to the blank, calibration standards and the sample. This is essential for the normalization of analyte signal to the internal standard signal. Plotting the ratio of analyte signal to the internal standard signal as a function of analyte concentration in the standards allows for quantification of the analyte in the standard. Use of an appropriate internal standard controls for variability in extraction, HPLC injection and ionization between samples and allows for differentiation of two standard points at the lower end of the curve that would have generated a nearly identical signal.

An ideal internal standard is a compound that closely resembles the chemical species to be quantified in a sample such that the effects of sample preparation and analysis should be the same for the internal standard as well as the analyte of

interest. However, the internal standard should have a retention time that is sufficiently different to be distinguished by the instrument used.

7-hydroxycoumarin-sulfate for sulfation reactions and 7-hydroxycoumarin-glucuronide were used as internal standards in this study. They were chosen because they ionized in negative mode as the analyte and had a retention time that was distinguishable from the analyte of interest.

5.4 Choice of metabolite standards

The standards were chosen such that they represent the metabolites expected to be formed *in vitro*. At positions 3 and 17 the carbon atoms in 17β/α-estradiol are bonded to hydroxyl group (OH). It is easier to deprotonate the hydroxyl group than it is to remove hydrogen from the hydrocarbon backbone of estradiol to allow for conjugation. This is in agreement with the literature where most of the conjugative metabolites of estradiol in various species and tissues were sulfated/glucuronidated at 3 and/or 17 position (Dunn, 1977; Maume et al., 2001). The standard metabolites would be subjected to the same chromatographic conditions as the test sample and hence would have a chromatographic profile that is similar to the metabolites in the test samples. These metabolite standards would then be used for quantifying unknown metabolites in the test samples based on similarity of retention time (RT).

The only difference between 17β-estradiol and 17α-estradiol is in the orientation of the 17 OH group. It was assumed that this difference was too minor to affect the chromatographic properties of the diastereoisomers and hence will not affect the RT time of its resultant metabolites It is for this reason that 17β-estradiol metabolite standards were used for studying 17α-estradiol metabolism.

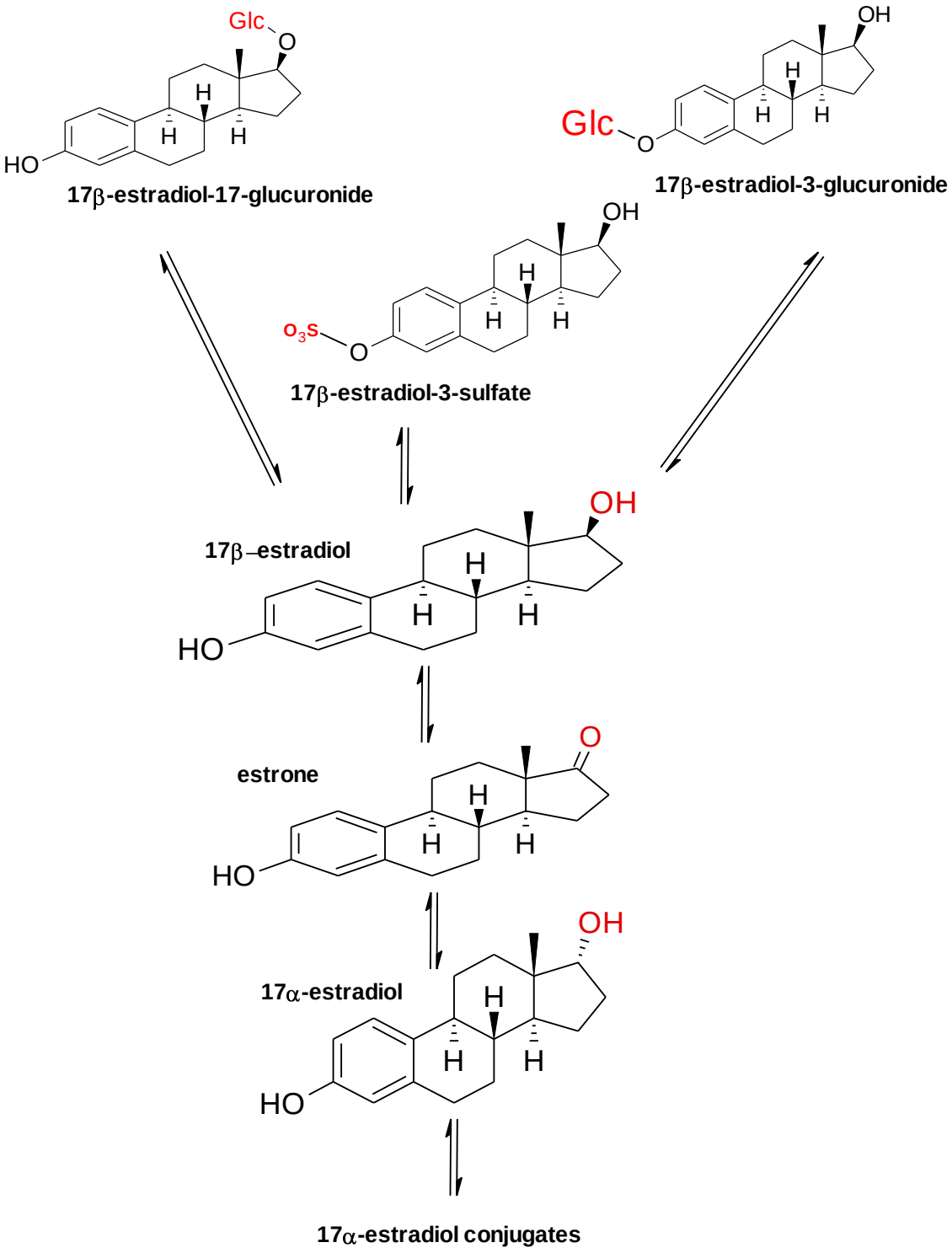


Figure 58: Conversion of 17β-estradiol to form 17α-estradiol and its conjugates.

17β-estradiol can also be directly conjugated to sulfate and or glucuronic acid groups. Shown in this diagram are the chemical structures of 17β-estradiol-3-sulfate, 17β-estradiol-3-glucuronide and 17β-estradiol-17-glucuronide used as metabolite standards for the identification and quantification of 17β/α-estradiol

5.5 Optimization of chromatographic conditions for the detection of metabolites of estradiol

Metabolite standards of 17β-estradiol-3-glucuronide (E3G), 17β-estradiol-17-glucuronide (E17G), and 17β-estradiol-3-sulfate (E3S) were spiked in phosphate buffer and loaded onto an appropriately chosen UPLC column. Conditions that resulted in the formation of clear distinct peaks of E3G, E17G, E3S and allowed the separation of metabolite standards from each other and their internal standard were chosen. E3G and E17G were separated on an acquity UPLC HSS T3; 1.8μm column using a 0.1% formic acid: acetonitrile (10 to 70%) gradient. E3G eluted before E17G at retention time of 2.56 and 2.75 respectively (figure 59). For 17β-estradiol reactions a single peak corresponding to the formation of E3G was seen at a retention time of 2.56 min. For 17α-estradiol reactions a single peak corresponding to 17α-estradiol-17-glucuronide was detected at a retention time of 2.72 min. For sulfation reactions, the metabolites in samples had a very low signal intensity and poor chromatography. After stopping the reaction with acetonitrile, the samples were centrifuged at a high speed in order to pellet the cytosol that could clog the column and interfere with the results. The supernatant containing acetonitrile was dried in a nitrogen dryer and resuspended in 0.1% aqueous formic acid before injecting it on to an Acquity UPLC BEH shield RP18; 1.7μm column. The internal standard 7-hydroxycoumarin sulfate elutes before the only metabolite E3S detected at a retention time of 2.01 min. The signal intensity observed is much lower than what was seen for glucuronidation reactions. (figure 60).

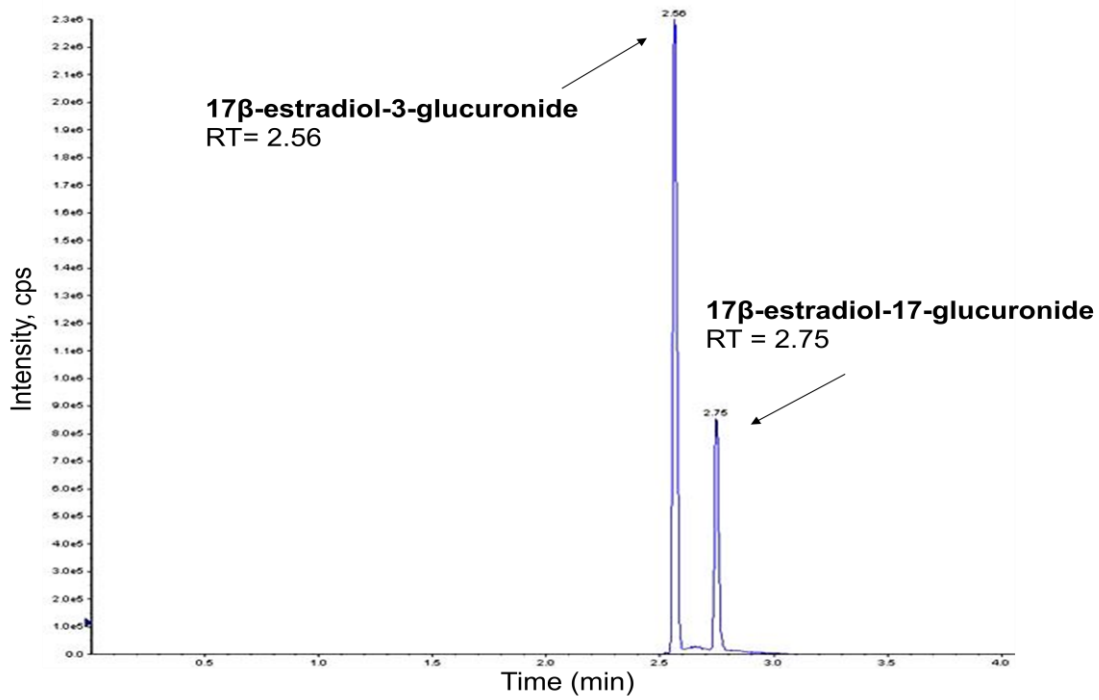


Figure 59: Chromatogram showing the separation of metabolite standards of 17β-estradiol.

17β-estradiol-3-glucuronide and 17β-estradiol-17-glucuronide at retention times (RT) 2.56 and 2.75 respectively.

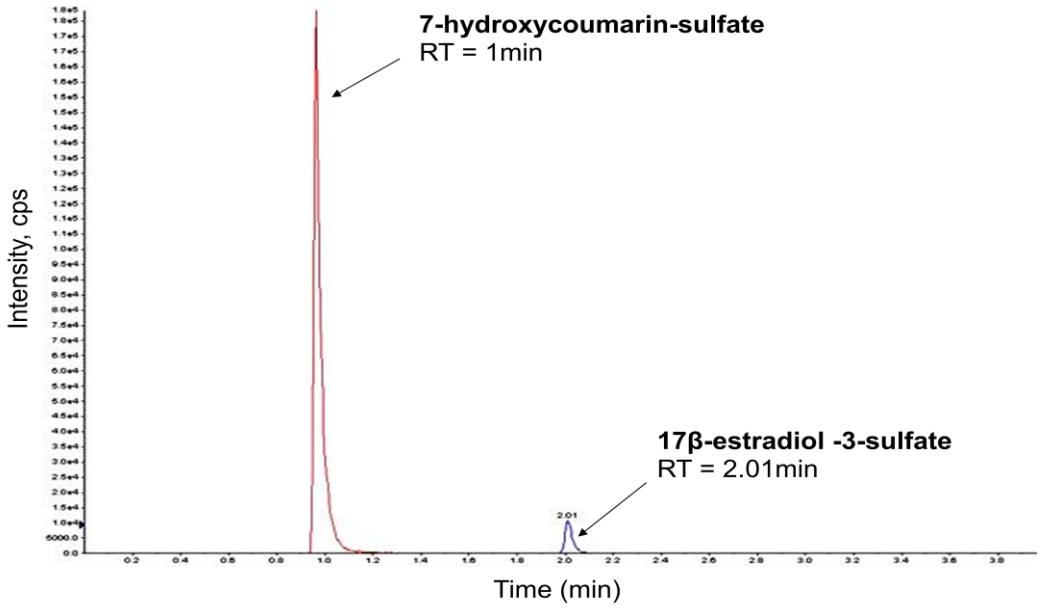


Figure 60: Chromatogram showing the detection of sulfate metabolites of 17β-estradiol and 7-hydroxycoumarin.

17β-estradiol-3-sulfate had a retention time of 2.01 min in bovine liver cytosol incubated with 17β-estradiol. The internal standard 7-hydroxycoumarin-sulfate elutes at 1min.

5.6 Rate of formation of 17β-estradiol-3-glucuronide in bovine liver microsomes

17β-estradiol was solubilized in 100% DMSO and serially diluted such that the final concentration of DMSO in the assay was at 5%. A study carried out by Dehal *et al* showed no significant change in activity of major UGT isoforms at 5% DMSO. (http://www.bdj.co.jp/gentest/articles/hkdqj2000001c8mr-att/Poster5-3_UGT_org_sol.pdf) A sample prepared by pooling 8 male liver microsomes (BD Gentest, 20mg/ml) was used for detection of 17β-estradiol metabolites followed by optimization of assay conditions. Assay conditions were optimized for incubation time and protein concentration prior to carrying out kinetic reactions for the metabolites formed. For these optimization reactions, formation of the metabolite was plotted against incubation time and protein concentration and only values that fell within the linear part of the curve were chosen. For protein optimization assays, protein concentration that consumed less than 10 % of the overall substrate present was used. All this was done to avoid performing kinetic reactions at saturating conditions. Standards contained a mixture of 17β-estradiol-3-glucuronide and 17β-estradiol-17-glucuronide at known concentrations. All incubations were carried out at 37°C in a 96 well plate. Reactions were started by the addition of UDPGA. UDPGA was not added to control wells. The metabolite production in the control sample was subtracted from metabolite production in the test samples. This was done to correct for any glucuronidation occurring due to the presence of some inherent UDPGA in bovine liver microsomes.

The V_{max} and the K_m (figure 61) were calculated by fitting the curve to Michaelis-Menten equation using GraphPad Prizm software. The Michaelis-Menten model of

single substrate reaction is based on the knowledge that enzyme catalysed reactions are saturable. The initial rate of reaction measured over a range of substrate concentration increases with increasing substrate concentration. However, as the active sites on the enzyme become saturated with the substrate, the rate of reaction becomes steady as it reaches a maximum called V_{max}. UGTs that catalyse glucuronidation reactions are high capacity enzymes. This explains the high velocity and V_{max} value seen. A low K_m value for the reaction indicates that the UGTs responsible for the formation of 17β-estradiol-3-glucuronide are indeed very efficient. On one hand, the formation of 17β-estradiol-3-glucuronide was noted to be a very efficient process that reached saturation levels very quickly, whereas on the other hand formation of 17β-estradiol-17-glucuronide was almost negligible.

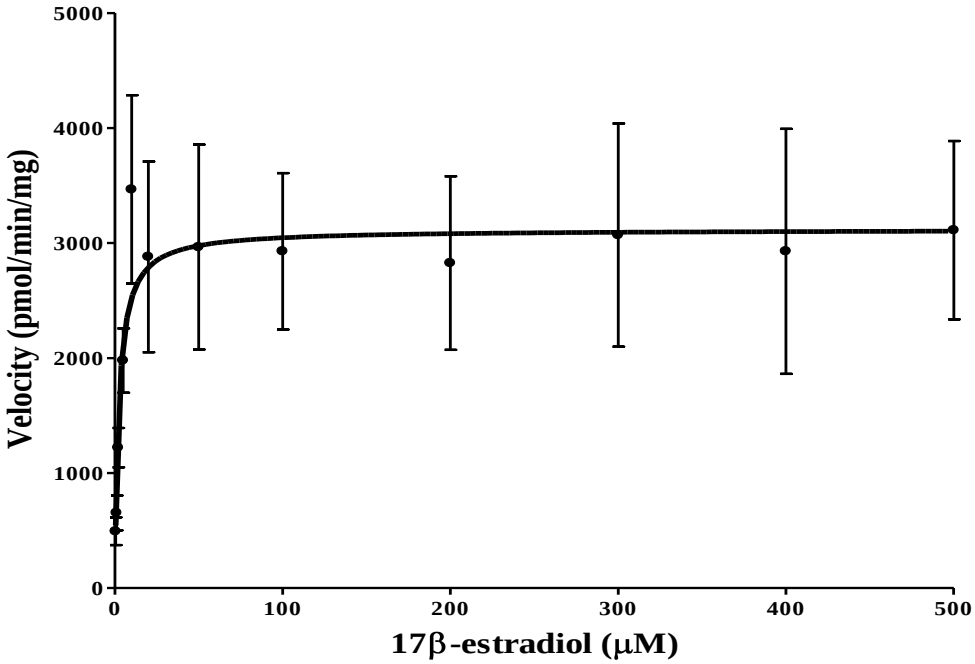


Figure 61: Kinetics of formation of 17β-estradiol-glucuronide in bovine male liver microsomes.

Rate of formation of 17β-estradiol-3 glucuronide as a function of 17β-estradiol activity in a pool of 8 male bovine liver microsomes at 0.05mg/ml-pH 7.4. The rate of reaction had a V_{max} of 3118 ± 159 pmol/min/mg and a K_m of 2.4 ± 0.7μM. Each assay was carried out in triplicate in a 96 well plate. Data points are average values of each assay that was carried out in triplicates ± standard error.

5.7 Rate of formation of 17α-estradiol-17-glucuronide in bovine liver microsomes.

Microsome incubations were carried out in a similar manner as 17β-estradiol incubations using 17β-estradiol-3-glucuronide and 17β-estradiol-17-glucuronide as standards. As opposed to glucuronidation of 17β-estradiol that produces 17β-estradiol-3-glucuronide, glucuronidation of 17α-estradiol produces the 17α-estradiol-17-glucuronide. Formation of the 3-glucuronide was not detected. Kinetics on the formation of 17α-estradiol-17-glucuronide was carried out in a substrate concentration range of 0-100 μM (figure 62). To get an accurate estimation of V_{max} and K_m, kinetic experiments are generally performed in a substrate range that encompasses the linear part of the curve where substrate concentration is directly proportional to the rate of metabolite formation and the part of the curve where increase in substrate concentration does not result in increase or causes inhibition of enzyme activity i.e. where saturation /inhibition occurs. It was not possible to achieve that in this case because of the solubility issues. 17α-estradiol was harder to solubilize compared to its β counterpart. It was used at a final concentration of 5% DMSO in the assay. Going above the substrate concentration of 100μM would mean using a higher percentage of DMSO to get 17α-estradiol completely in solution. It was thought that using a higher percentage of DMSO could have unforeseeable adverse effects on the activity of UGTs responsible for estradiol glucuronidation. The rate of formation of 17α-estradiol-17-glucuronide had a very high K_m and a low V_{max} meaning that it was less efficient compared to its 17β-estradiol counterpart.

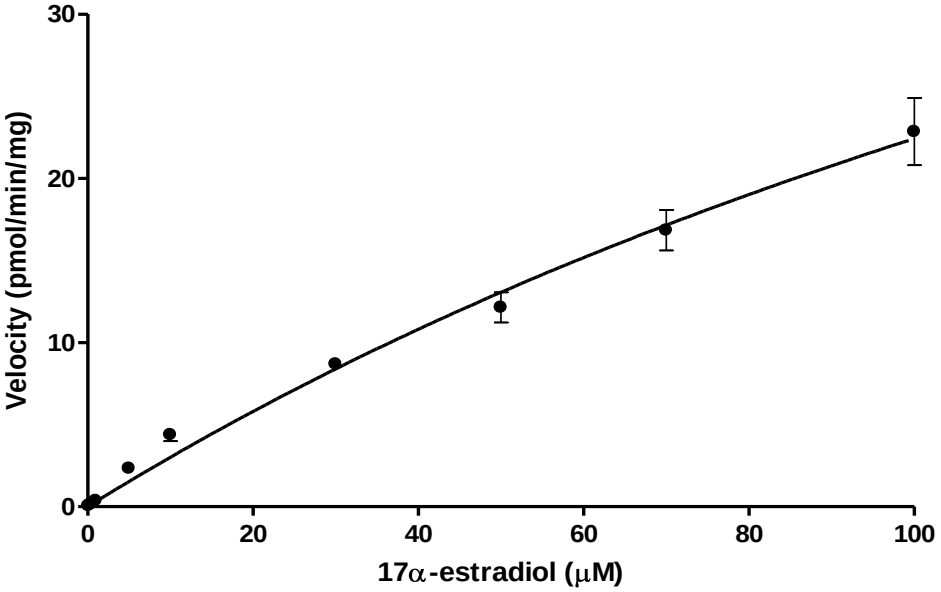


Figure 62: Kinetics of formation of 17α-estradiol-glucuronide in bovine male liver microsomes.

Rate of formation of 17α-estradiol-17glucuronide as a function of 17α-estradiol activity in a pool of 8 male bovine liver microsomes at 0.05mg/ml-pH 7.4. The rate of reaction had a V_{max} of 79 ± 17 pmol/min/mg and a K_m of 252 ± 71μM. Each assay was carried out in triplicate in a 96 well plate. Data points are average values of each assay that was carried out in triplicates ± standard error.

5.8 Rate of formation of 17β-estradiol-3-sulfate in female bovine liver S9

Negligible amount of sulfation was observed in the male bovine liver cytosol. The level of metabolite formation was too low for carrying out kinetics. S9 fraction from bovine female liver was found to produce 17β-estradiol-3-sulfate at levels that allowed quantitation. Hence it was decided to use bovine female liver S9 for kinetics. The S9 fraction is a supernatant fraction obtained by centrifuging an organ (usually liver) homogenate in a suitable medium at 9000g for 20 min. This fraction contains an entire set of drug metabolizing enzymes in the cytosol (Sulfotransferses) and microsomal (Cytochrome P450 and UGTs) components. No sulfate metabolites of 17α-estradiol were detected.

The formation of 17β-estradiol-3-sulfate has a V_{max} of 1.9 pmol/min/mg, a K_m of 22.5nM and a inhibition constant, K_i of 246nM. These were calculated by fitting the values to a partial substrate inhibition curve since substrate inhibition was observed at concentrations above 0.1μM (figure 63). A low K_m is indicative of the high affinity that SULTs have towards their substrates. Low K_m (nM range) values for 17β-estradiol sulfation in liver cytosol has been observed in humans (Kester et al., 1999b; Song and Melner, 2000)

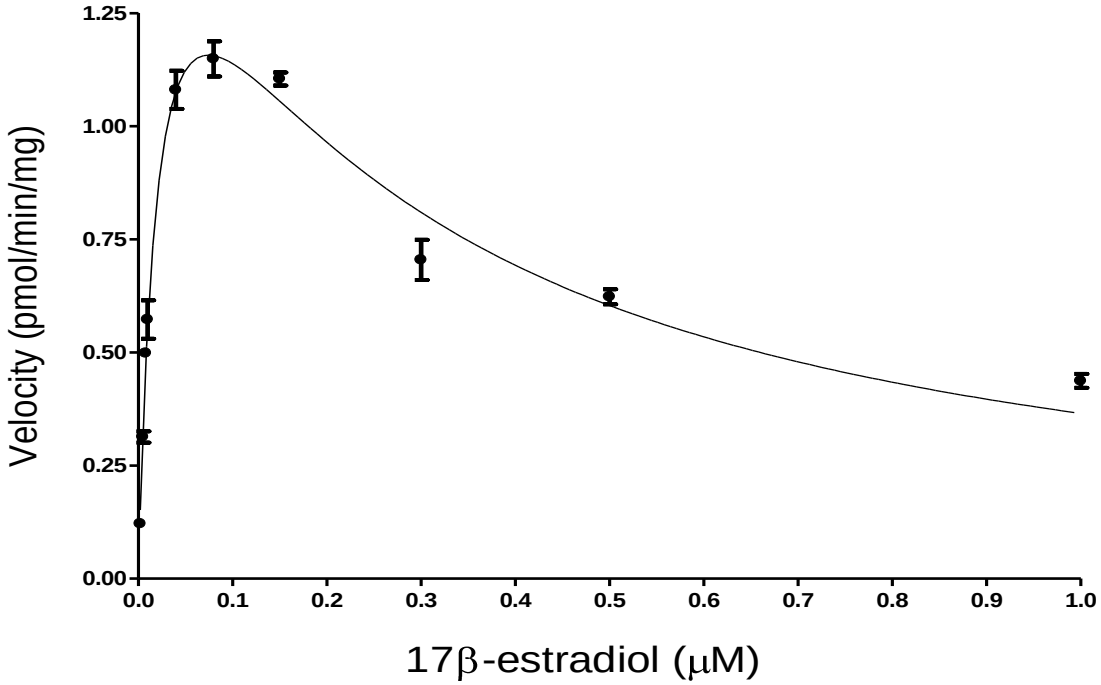


Figure 63: Kinetics of formation of 17β-estradiol-sulfate in bovine female liver S9.

Rate of formation of 17β-estradiol-3-sulfate as a function of 17β-estradiol activity in bovine female liver S9. The rate of reaction had a V_{max} of 1.9 ± 0.1 pmol/min/mg and a K_m of 0.02 ± 0.003 μM. Ki was 0.25 ± 0.03 Each assay was carried out in triplicate in a 96 well plate. Data points are average values of each assay that was carried out in triplicates ± standard error.

Metabolite	Source	V_{max} (pmol/min/mg)	K_m (µM)	K_i (µM)
17β-estradiol-3-glucuronide	Bovine male liver microsmes (pool)	3118 ± 159	2.4 ± 0.7	N/A
17α-estradiol-17-glucuronide	Bovine male liver microsmes (pool)	79 ± 17	252 ± 71	N/A
17β-estradiol-3-sulfate	Bovine female liver S9	1.9 ± 0.1	0.02 ± 0.003	0.25 ± 0.03

Table 36: Summary of rate of formation of Estradiol metabolites in bovine liver in vitro.

Kinetics was carried out on a pool of 8 bovine male liver microsomes for glucuronidation reactions and on bovine female liver S9 for sulfation reactions. Each assay was carried out in triplicate in a 96 well plate. Values obtained are average of the triplicates ± standard error.

5.9 Species and sex differences in the sulfation of 17β-estradiol from liver cytosol and S9 fraction.

In order to see how sulfation of 17β-estradiol in other species related to that in cattle, it was decided to perform a qualitative analysis on cytosol and S9 obtained from the liver of different species and sex (where possible). Assays were carried out using 5, 50 and 500nM concentrations of 17β-estradiol (figure 64 and 65). These concentrations were chosen based on a literature search which revealed that humans sulfate 17β-estradiol in the nanomolar range (Kester et al., 1999b). Overall, sulfation in S9 had lower velocity values compared to that in cytosol. S9 fraction contains a diluted pool of sulfotransferases as opposed to cytosol, hence the difference. Cat liver extensively sulfated 17β-estradiol as seen in cytosol and S9. It is known that due to the absence of certain UGT enzymes such as UGT1A6, the cat family cannot perform certain glucuronidation reactions (Court MH, 2000). It is quite possible that they heavily depend on sulfation for metabolism of

xenobiotic compounds. However this assumption is entirely speculative. Minor differences were noted in the cytosolic sulfation of 17β-estradiol in male and female cats. No significant sulfation was observed in dog liver and male bovine liver in both cytosol and S9. Sex related differences were seen in rat liver cytosol and bovine liver S9. Expression of estrogen sulfotransferase in rats is known to be regulated in an age and sex specific manner where young male rats have a higher expression of the enzyme compared to the female rats (Demyan WF, 1992; Song W-C, 1997). This is in agreement with what has been observed here where the activity in the male rat liver cytosol is much higher than the female rat liver cytosol due to higher expression levels of estrogen sulfotransferase in the male rat. Substrate inhibition at increased 17β-estradiol concentration was observed only with the bovine female liver S9 and the bovine male liver cytosol. Substrate inhibition of estrogen sulfotransferase has also been reported in the male rat liver with concentration of 17β-estradiol above 1µm (Falany et al., 1995c)

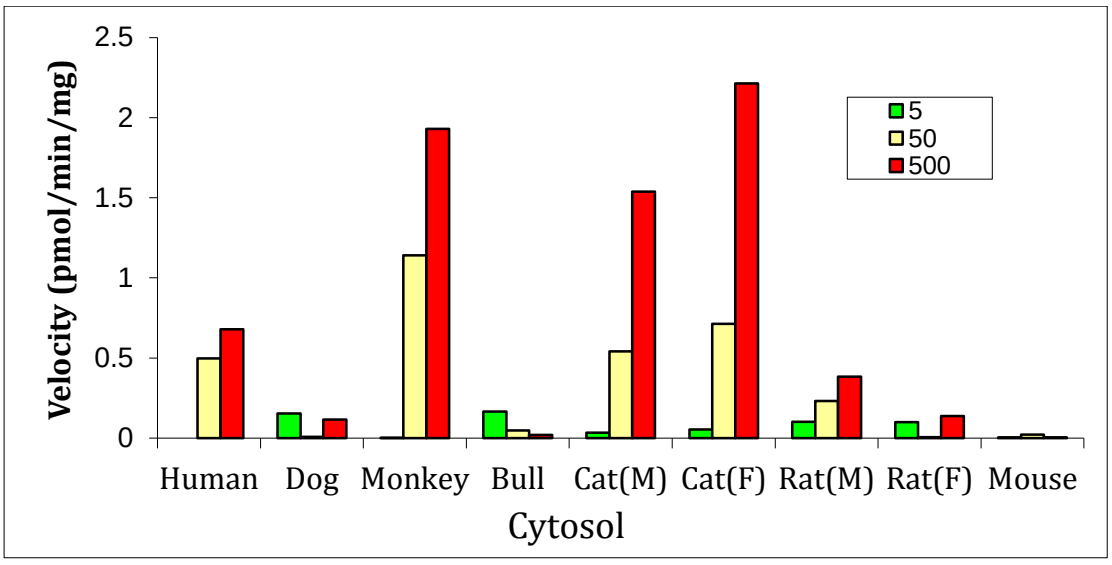


Figure 64: 17β-estradiol sulfation in liver cytosol across different species and sex

Formation of 17β-estradiol-3- sulfate as a function of 17β-estradiol activity at 5, 50 and 500nM in liver cytosol from different species and sex.

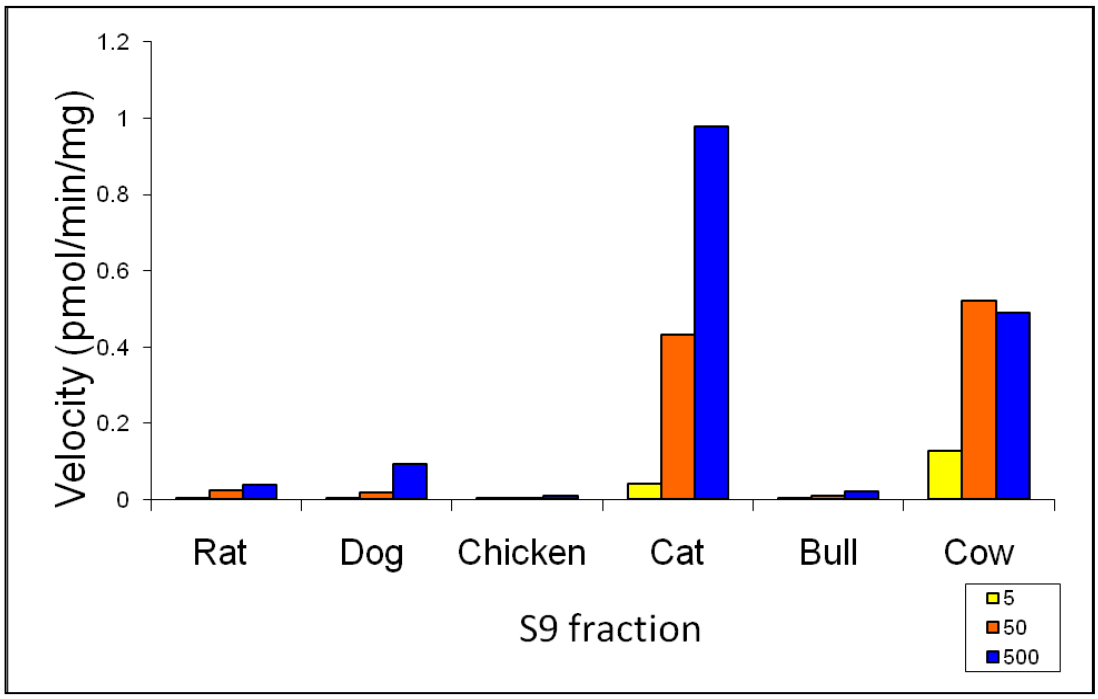


Figure 65: 17β-estradiol sulfation in liver S9 across different species

Formation of 17β-estradiol-3-sulfate as a function of 17β-estradiol activity at 5, 50 and 500nM in S9 fraction from liver of different species.

5.10 Assessing cell viability of cryopreserved bovine hepatocytes over a period of time

Upon removal from liquid nitrogen, cell viability of cryopreserved hepatocytes was assessed using trypan blue exclusion method. The cells were counted on an automated BIO RAD cell counter and had a viability of 86%. In order to assess viability over a period of time the cells were incubated with 5% DMSO as in the test incubation and left at 37°C. Aliquots were withdrawn at 0, 3 min, 2.5 and 4 hour upon incubation and cells counted on the cell counter. It was found that after 3 minutes, there was no difference in the cell viability compared to the start. Owing to the large production of glucuronide conjugates seen in a short period of time with hepatocytes and microsomes, an incubation time of 3 minutes was

applied to all kinetic reactions here. After 2.5 hours, the cells had only 33% viability which further dropped to 18% after 4 hours (figure 66).

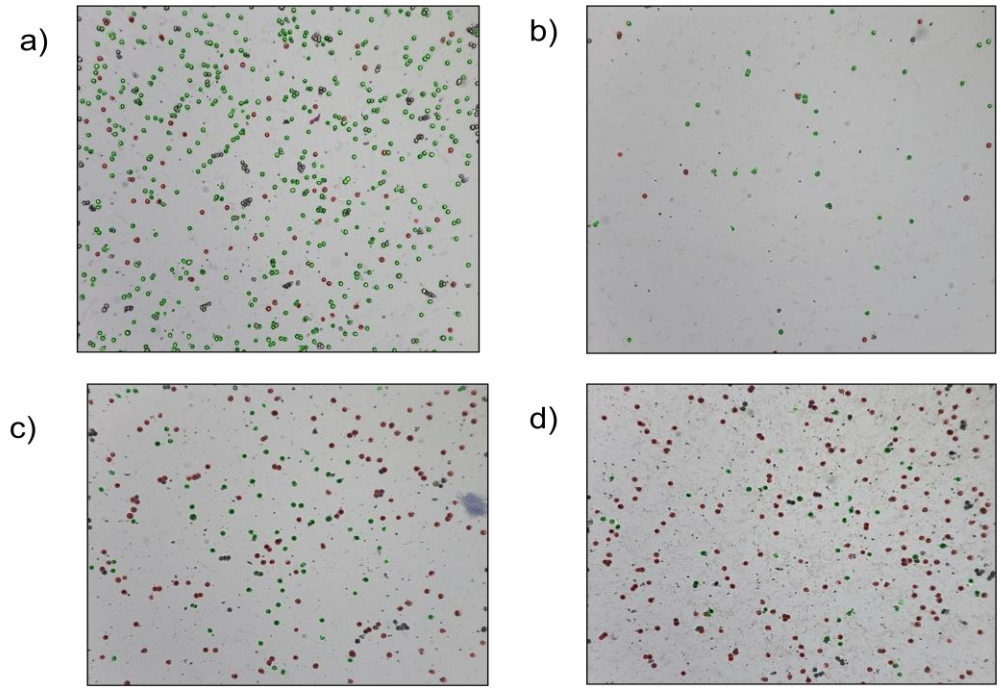


Figure 66: Cell viability of cryopreserved hepatocytes assessed over a period of time using trypan blue exclusion methods.

Cryopreserved hepatocytes upon removal (5.10a) have a very high viability of 86%. No significant difference was seen in cell viability after a 3 minute incubation; 5.10b. After 2.5 hours the cell viability drops to 33%; 5.10c followed by a further drop to 18% after 4 hours; 5.10d after removal from cryo conditions. The cells were incubated with 5% DMSO to mimic incubation conditions. Green circles indicate viable cells and red ones are the dead cells.

5.11 Identification of 17β-estradiol metabolites upon incubation of 17β-estradiol with cryopreserved bovine hepatocytes.

In order to check for the presence and if possible identify any metabolites formed, some preliminary incubation with 17β-estradiol was carried out. 3 major peaks were detected at retention time of 2.56, 2.72 and 2.81 of which the peak at 2.56 and 2.72 corresponded to 17β estradiol-3-glucuronide and 17β-estradiol-17-glucuronide respectively. A neutral loss scan confirmed their identity. The identity of the metabolite giving rise to the 2.81 peak was unknown (figure 67).

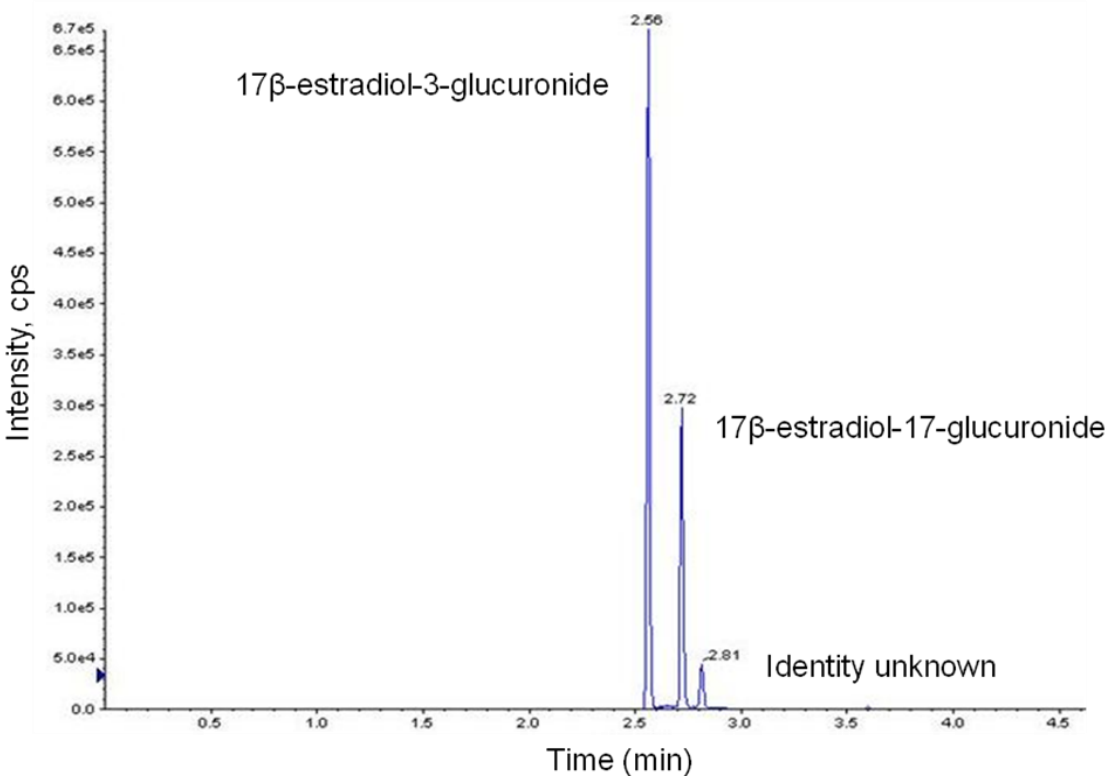


Figure 67: Chromatogram showing metabolites of 17β-estradiol detected in cryopreserved bovine hepatocyte incubations.

17β-estradiol-3-glucuronide was the one to elute first at 2.56 min followed by 17β-estradiol-17-glucuronide at 2.72 min. The identity of the metabolite eluting at 2.81 min was unknown. Formation of 17β-estradiol-3-sulfate was not detected.

5.12 Rate of formation of 17β-estradiol-3-glucuronide as a function of 17β-estradiol in bovine hepatocytes

Hepatocytes at a final concentration of 1 x10^6 cells/ml in Hepatocyte Maintenance Media (HMM) buffer were used in the assay. Kinetics was carried out on the formation of 17β-estradiol-3-glucuronide in a similar manner to the one done in microsomes. Velocity of the reaction was measured in pmol/min/10^6 cells. A K_m of 1.2 μM (figure 68) was seen which is not very different from the K_m of 2.36 μM seen in microsomes. This suggests that probably the same sets of UGT enzymes are responsible for the metabolism of 17β-estradiol in microsomes and hepatocytes. It also implies that concentration of 17β-estradiol achieved in hepatocytes is equivalent to levels of 17β-estradiol available to UGTs in microsomes. This is given the fact that drug transporters are not present in microsomes. An interesting phenomenon observed was that as the production of 17β-estradiol-3-glucuronide rose the formation of 17β-estradiol-17-glucuronide fell (figure 69). Microsomes contain UGTs as do hepatocytes but they do not contain drug transporters that are present in hepatocytes. Drug transporters are vital since they can affect drug metabolism by altering accessibility of the drug to drug metabolizing enzymes. Inhibitors of these transporters can also affect drug concentrations reached *in vivo*. A possible hypothesis for the observed phenomenon is that 17β-estradiol-3-glucuronide formation inhibits the uptake of further 17β-estradiol thus affecting the formation of 17β-estradiol-17-glucuronide by certain UGTs. A complex interplay between drug metabolizing enzymes and transporters is probably at work here.

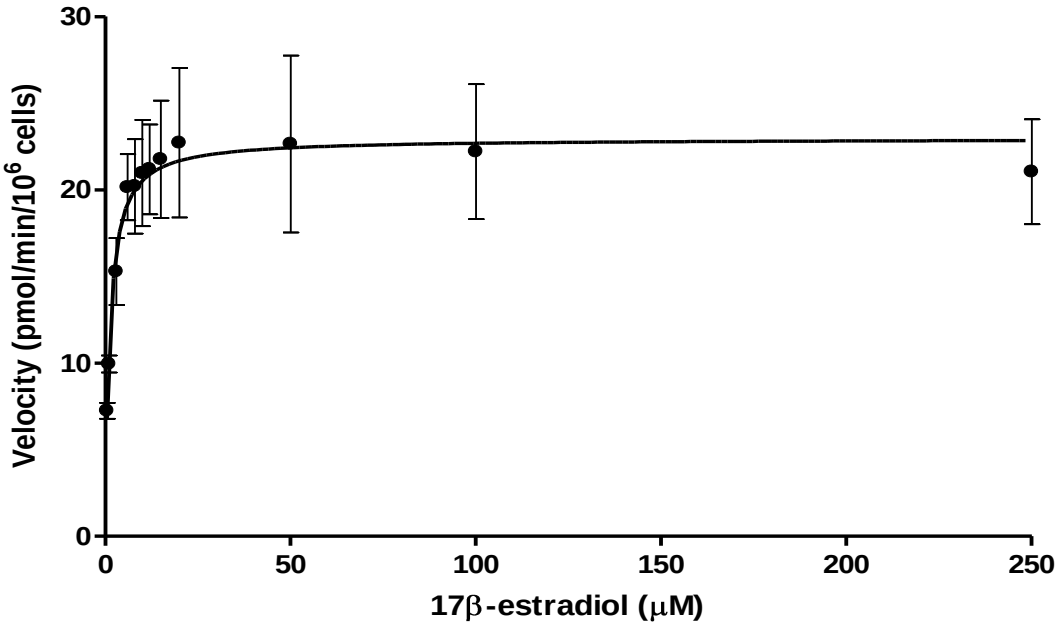


Figure 68: Kinetics of formation of 17β-estradiol-glucuronide in cryopreserved bovine hepatocytes

Rate of formation of 17β-estradiol-3-glucuronide as a function of 17β-estradiol in bovine hepatocytes. The rate of reaction had a V_{max} of 23 ± 0.7 pmol/min/10^6 and a K_m of 1.2 ± 0.2 μM. Each assay was carried out in triplicate in a 96 well plate. Data points are average values of each assay that was carried out in triplicates ± standard error.

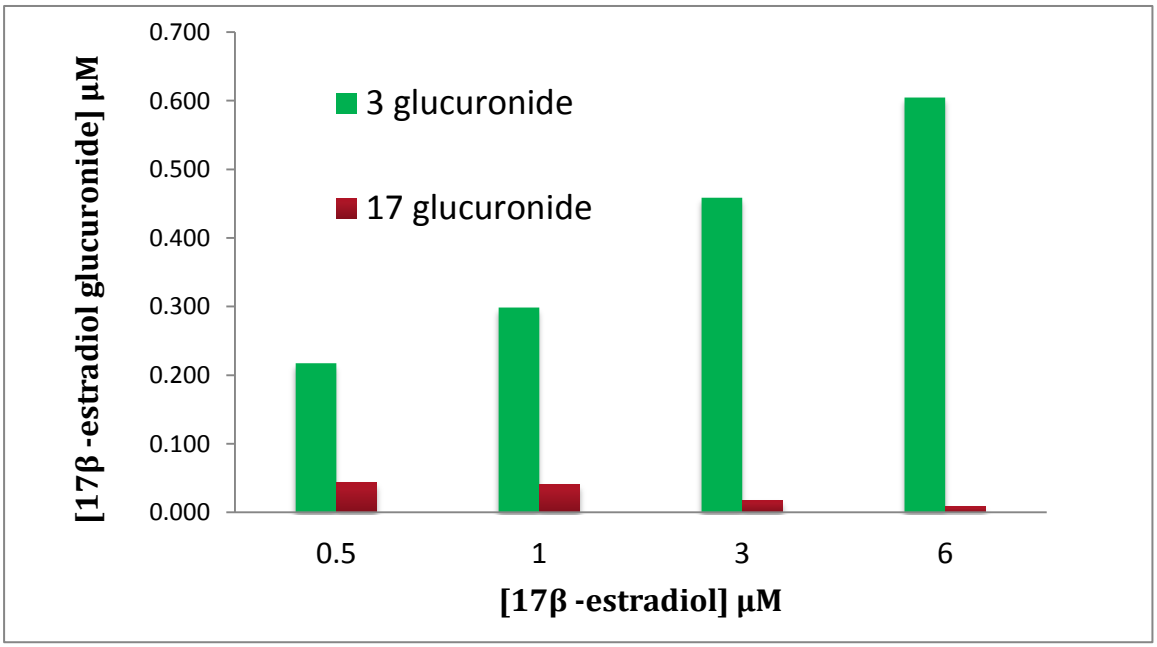


Figure 69: Formation of 17β-estradiol glucuronides in cryopreserved bovine hepatocytes

Relative formation of 17β-estardiol-3-glucuronide and 17β-estradiol-17-glucuronide at increasing concentration of 17β-estradiol in bovine hepatocytes.

5.13 Discussion

5.13.1 **17α Vs. 17β-estradiol**

The majority of ruminants including cattle undergo oxidoreduction that converts 17β-estradiol to estrone and estrone to 17α-estradiol. The 17α-estradiol that is formed is preferentially glucuronidated. Knowing this it was interesting to compare the metabolite formation and the rate of glucuronidation of the two diastereoisomers. We found that 17β-estradiol was mainly glucuronidated at the 3-OH position whereas 17α-estradiol was preferentially glucuronidated at the 17-OH position. The glucuronidation of 17β-estradiol was a more efficient reaction with a lower K_m and high reaction rate than the glucuronidation of 17α-estradiol. This could in part be explained by the fact that it is easier to conjugate glucuronic acid on to the hydroxyl group that is part of an aromatic hydrocarbon (3-OH) than it is to do so with a hydroxyl group that is a part of an aliphatic ring (17-OH). Several different UGT enzymes catalyse the glucuronidation of estradiol but vary significantly in their kinetics, stereoselectivity and regioselectivity (Itaaho et al., 2008). The majority of the members of the UGT1A family conjugated one or both the diastereoisomers at the 3-OH position with the exception of UGT1A4 whose activity was low and uniquely directed towards the 17-OH of both aglycones. The UGT2B family usually conjugated both estradiols at the 17-OH position with varying stereoselectivities. For example UGT2B4 is specific for epiestradiol whereas UGT2B7 can glucuronidate both the stereoisomers whilst UGT2B17 specifically glucuronidated 17β-estradiol (Itaaho et al., 2008). Expression of these enzymes can be tissue age and sex specific. They can also vary between species. Rabbit, pig, bovine and elk liver microsomes preferentially glucuronidate both distereoisomers at the 3-OH position whereas 17α-estradiol-17-glucuronide and 17β-estradiol-17-glucuronide were the major metabolites in human and rat liver

microsomes respectively (Itaaho et al., 2008). 17β-estradiol-3-glucuronide was the major metabolite detected in our study, however contrary to the results obtained in this study we did not detect the presence of 17α-estradiol-3-glucuronide. Differences in kinetics of the two diastereoisomers depend on the expression profile of specific UGTs for the tissue age sex and species tested.

5.13.2 **Hepatocytes as a model for studying drug metabolism**

Hepatocytes contain both phase 1 and phase 2 drug metabolizing enzymes along with the necessary cofactors in concentrations that are representative of *in vivo* conditions. In addition to this they also contain drug transporters and hence able to achieve *in vivo* like drug concentration in cells. These properties make hepatocytes a good *in vitro* model for *in vivo* drug metabolism. In comparison to microsomes, hepatocytes are known to predict metabolic clearance more accurately (Brown et al., 2007; Lam and Benet, 2004). Freshly isolated hepatocytes are considered as the best *in vitro* model for studying drug metabolism in the liver because of their ability to closely mimic liver cells. However, there are several limitations to their successful usage. A major hindrance is the loss of expression and/or activity of the major drug metabolizing enzymes such as cytochrome P450s (CYPs), UGTs and SULTs (Li et al., 1999a). A study carried out by Liu *et al*, 1996 on primary rat hepatocyte cultures showed significant decline in the expression of major SULT isoforms such as SULT1A1, SULT1C1, SULT1E1 and SULT2A1 within first 24 hours of hepatocyte isolation (Liu et al., 1996). This makes it very difficult to arrange logistics and plan experiments in a short period of time after hepatocyte isolation. Hence cryopreservation of hepatocytes has evolved as a lucrative means of preserving hepatocytes for long term usage. Some initial studies have shown that viability

and yield of cryopreserved hepatocytes from several different species was only slightly reduced after cryopreservation (Li et al., 1999b). No significant difference was observed in the drug metabolizing enzyme activities between freshly isolated and cryopreserved hepatocytes for the major drug metabolizing pathways. We detected the presence of 17β-estradiol-3-glucuronide in both microsomes and hepatocytes. No 17β-estradiol-17-glucuronide was detected in either of the two. Kinetics of the formation of 17β-estradiol-3-glucuronide was similar in both the model systems. Similar K_m values indicated that probably the same sets of UGTs were metabolizing 17β-estradiol in microsomes and hepatocytes. Drug transport activity too seemed to be maintained in hepatocytes. Velocity of the glucuronidation reaction was not comparable since in microsomes velocity was measured in pmol/min/mg of total protein whereas in hepatocytes, velocity was measured in pmol/min/1x10^6cells. Similarly 17α-estradiol-17-glucuronide was produced in both microsomes as well as hepatocytes. Kinetics was not performed on the formation of 17α-estradiol-17-glucuronide since increase in this metabolite over time was not observed in hepatocytes. One possible explanation for this could be that some fraction of 17α-estradiol-17-glucuronide generated was being deglucuronidated by glucuronidases present in the lysosome. Low amount of 17β-estradiol-3-sulfate was produced from 17β-estradiol in bovine female liver S9, however no 17β-estradiol-3-sulfate was detected in female liver hepatocytes. Limited information is available to evaluate the effects of cryopreservation on gene expression and responsiveness to hormones. It has been noted that cryopreservation affects CYPs that bring about metabolism of the steroid androst-4-ene-3,17-dione in dog hepatocytes. Similarly, cryopreserved dog hepatocytes also lose their ability to respond to α1 and β2 adrenergic receptors indicating possible damage to receptors and/or their transduction

system (Skett et al., 1999) Expression of the estradiol metabolizing SULT1E1 is also tightly regulated by hormonal activity in humans. (Rubin et al., 1999). Hence any damage during cryopreservation affecting the regulation of SULT1E1 can result in its loss of expression and activity in bovine hepatocytes.

5.13.3 **Glucuronidation versus sulfation in bovine liver**

Clearly, glucuronidation seems to be the most important pathway responsible for the metabolism of estradiol in bovine liver. The `results here are in agreement with observations noted *in vivo* where majority of estradiol metabolites detected have been glucuronide conjugates (Maume et al., 2001). No sulfate conjugates have been detected in *vivo* in the bovine liver (Maume et al., 2001). It is quite possible that sulfate conjugation might play a role in extra hepatic metabolism of estradiol especially in hormone responsive tissues such as the endometrium where it might be responsible for mediating important biological functions (Rubin et al., 1999).

6. Concluding Remarks

6.1 Summary

Significant advances have been made in sulfotransferase research in the recent years with respect to understanding the structural features that govern activity and specificity. Since sulfotransferases are recognised as a major xenobiotic drug and steroid metabolising pathway, profiling the quantity and activity of SULTs in major drug metabolising tissues has allowed better prediction of drug toxicity. However, most of the research has predominantly being carried out in humans. Administration of veterinary drugs and other xenobiotics to cattle can often result in the accumulation of toxic metabolic residues in edible tissues that can potentially affect humans through the food chain. An understanding of drug metabolism in this species is therefore vital to ensure safe use of drugs in cattle and eventually the provision of safer animal food products to man. Despite this very little research has been done on drug metabolising enzymes in cattle. This study has produced useful tools such as recombinant enzymes for studying some of the major drug metabolising isoforms of SULTs in cattle. Valuable insights have been generated into the sulfation of steroids and xenobiotics in the bovine liver. In addition to this, conjugative metabolism of the steroid hormone 17β-estradiol and its diastereoisomer 17α-estradiol was investigated in cattle using different model systems such as cytosol, microsomes and hepatocytes.

6.2 Major findings

Like human SULT1B1, bovine SULT1B1 also had overlapping substrate specificities with SULT1A1. Substrate specificity profiling carried out with equal amount of bovine SULT1A1/SULT1B1 revealed that bovine SULT1B1 was more efficient in the sulfation of small phenolic compounds compared to bovine SULT1A1. This is in contrast to humans where sulfation brought about by SULT1A1 is several orders of magnitude higher than SULT1B1. Bovine SULT1A1

also demonstrated substrate inhibition with 4-nitrophenol like human SULT1A1. However, it was less intense (K_i is 24μM) than human SULT1A1 (K_i is 34μM) (Riches et al., 2007). In male and female bovine livers, the expression of SULT1B1 was found to be higher than SULT1A1. Whilst the male livers displayed Michaelis-Menten kinetics with 4-nitrophenol, the female livers demonstrated substrate inhibition. 4-nitrophenol is sulfated by many members of the SULT1 family with overlapping substrate specificities. The difference in kinetics of 4-nitrophenol between male and female bovine liver suggests that different members of the SULT1 family are involved in 4-nitrophenol sulfation.

In humans, SULT1E1 has a very high affinity for the endogenous compound 17β-estradiol and is known to metabolise it in the low nanomolar range (Kester et al., 1999b). However, it was not found to be true for bovine SULT1E1 which had a higher K_m and metabolised 17β-estradiol in the micromolar range. Comparison of primary amino acid sequences of human and bovine SULT1E1 revealed major substitutions in the enzymes active site which could possibly account for some of the differences above. Furthermore, huge variation was seen in SULT1E1 expression in the bovine liver. The variation in expression observed was consistent with the activity detected with 17β-estradiol. An overall, low level of activity was observed with 17β-estradiol in bovine liver. Evaluation of microsomes, cytosol and hepatocytes for the conjugative metabolites of 17β-estradiol revealed that glucuronidation was probably the major pathway for 17β-estradiol metabolism in cow as most of the metabolites detected were glucuronide conjugates as opposed to the almost negligible amount of sulfate conjugates seen. Livers from cows treated with an exogenous progestin on average showed three fold increases in activity as compared to the untreated

ones. However, this finding cannot be attributed to the effect of the exogenous progestin because of the small sample size of 4 livers and the huge amount of variation seen in the expression and activity of SULT1E1 in between those treated livers.

Our untagged recombinant bSULT2A1 was insoluble; however soluble expression was achieved upon tagging the protein with maltose binding protein (MBP) but no activity against DHEA, androsterone or pregnenolone (SULT2A1 substrates) was detected. SULT2A1 was detected in bovine liver using anti human SULT 2A1 antibody however, no activity towards its probe substrate DHEA was detected. Activity was detected in the bovine liver with pregnenolone which is known to be metabolised by both SULT2A1 and SULT2B1 in humans (Strott, 2002).

6.3 Does DHEA have a greater role than DHEA sulfate in cattle?

The steroid, DHEA and its sulfo conjugate DHEA sulfate (DHEAS) are secreted by the adrenal cortex. DHEA and DHEAS are the most abundant circulatory steroids in humans and usually are precursors to the production of androgens and estrogens (Labrie et al., 2005). They are associated with numerous biological actions such as stress response, lipid metabolism, immune system activation and pathological phenomena such as behavioural and neurodegenerative disorders in humans (Hansen et al., 1997; Labrie et al., 2005). SULT2A1 is the major enzyme responsible for the sulfoconjugation of DHEA to DHEAS. It was unable to express recombinant bSULT2A1 in solution. Recombinant bSULT2A1 was expressed in solution as a fusion with MBP. However, no activity was detected against known SULT2A1 substrates. SULT2A1 was detected in bovine liver using anti-human SULT2A1 antibody, however no activity with DHEA was recorded. Comparison of the primary amino acid sequence of bovine and human SULT2A1 revealed major

substitutions in the active site region of bovine SULT2A1 which could possibly affect the secondary structure and function of the enzyme significantly. In female bovine, the mammary gland utilises DHEA to make androstene-3β, 17β-diol (Belvedere et al., 1996), a metabolite with immunoenhancing activity (Loria et al., 1996). Recently, it was also shown that cows with inflammatory foot lesions showed a 23% decrease in serum DHEA concentrations suggesting the importance of DHEA in the inflammation process in cattle (Almeida et al., 2008). However, in cows circulating DHEAS was found to be significantly lower than DHEA (Feher et al., 1977; Marinelli et al., 2007). Indeed, it is quite a possibility that DHEA as compared to DHEAS might have a bigger biological function in cows. This is reflected by the inability of SULT2A1 to sulfate DHEA in bovine which restricts the creation of large reservoirs of DHEAS.

6.4 17β-estradiol metabolism in the bovine liver

17β-estradiol is routinely used by the animal industry for growth promotion in cattle. Several studies have been carried out to detect the metabolites of 17β-estradiol *in vivo*. Results reveal that very few metabolites detected were sulfate conjugates and the majority of them were glucuronide conjugates (Ivie et al., 1986; Maume et al., 2001). This suggests a strong role for glucuronidation in the elimination of 17β-estradiol. Indeed, 17β-estradiol-3-glucuronide was the major metabolite formed in bovine liver microsomes and hepatocytes when detected using UPLC-MS. A large amount of variation was observed in the expression of SULT1E1 in the bovine especially in the female liver. In humans, expression of SULT1E1 in the endometrium is exquisitely regulated during the menstrual cycle under the influence of progesterone (Falany and Falany, 1996b). Since all the female livers were of the same breed and age, it is quite possible that the

variation seen was due the heifers being at different points in their menstrual cycle at the time of slaughter.

6.5 Is SULT1B1 the major xenobiotic metabolising enzyme in the bovine liver?

Recombinant bSULT1A1 was not very efficient in sulfation as compared to human SULT1A1. It sulfated phenolic compounds at a rate which was 10 times lower compared to human SULT1A1. The K_m for recombinant (non purified) bovine SULT1A1 sulfation of 4-nitrophenol was 33 μM as opposed to that of 4μM for human SULT1A1 (purified) (Riches et al., 2007). Lesser substrate inhibition with 4-nitrophenol was observed for bovine SULT1A1. A Possible explanation for these observations lie in the structural differences of the active site of human and bovine SULT1A1 that bind the second molecule of 4-nitrophenol. On the other hand, recombinant bovine SULT1B1 was found to be better at sulfating phenolic compounds than bovine SULT1A1 and had huge overlapping substrate specificity with bovine SULT1A1. SULT1B1 was detected in bovine liver whereas SULT1A1 was not. 4-nitrophenol sulfation by recombinant bovine SULT1B1 followed the Michaelis-Menten kinetics with no inhibition whereas that of recombinant bovine SULT1A1 followed Michaelis-Menten with partial substrate inhibition. No substrate inhibition was seen for 4-nitrophenol sulfation in bovine male liver as it followed Michaelis-Menten kinetics similar to that of recombinant bovine SULT1B1. These findings point towards the possibility that SULT1B1 rather than SULT1A1, might be the major xenobiotic metabolising enzyme in the bovine liver. Further evidence is needed to back up this hypothesis. Availability of a SULT1B1 specific probe substrate that is exclusively metabolised by SULT1B1 would certainly be of a great advantage.

6.6 Future work

6.6.1 Validation of structure function relationship

Our study has generated important tools in the form of recombinant SULTs for the study of conjugative drug metabolism in cattle. Comparison of primary and tertiary structure of bovine and human sulfotransferases threw light on prominent structural features that could possibly govern some of the functional differences observed in SULT activity between the human and bovine. Site directed mutagenesis of critical residues in the structure of bovine SULTs to resemble that of human SULTs will be required to establish whether the residues identified are responsible for making significant changes to SULT activity in cows. This information would certainly be useful to the pharmaceutical/animal health industry who would be able to better predict the fate of drugs metabolised by SULTs in cattle.

6.6.2 The need for a SULT1B1 probe substrate

Currently the research in the field of sulfotransferases is seriously limited by the availability of exclusive probe substrates to assess activity of individual isoforms in a tissue where more than 1 isoform of SULT is present. For example, 4-nitrophenol is metabolised by a range of members of the SULT1 family and was used in this study as a substrate to assay the activity of SULT1A1/1B1. We presented evidence which might suggest that SULT1B1 is more active in sulfation in the bovine liver as opposed to SULT1A1, which is known to be a major xenobiotic metabolising enzyme in human liver. However, in order to confirm this hypothesis we need a SULT1B1 probe substrate which to date has not been discovered due to the reason that SULT1B1 has a high overlapping substrate specificity with SULT1A1.

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